TISSUE FACTOR ANTIBODIES AND USES THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The invention relates to antibodies capable of binding to tissue factor, without inhibiting normal tissue factor mediated blood coagulation, and methods of making and methods of use thereof, including in the treatment of cancer.

Related Art

[0002] Tissue factor (TF) is a cell-anchored component that, together with factor VIIa, initiates blood coagulation in vivo. TF is a transmembrane glycoprotein with a 219 amino acid residue extracellular region, a 23 amino acid residue transmembrane region and a 21 amino acid residue cytoplasmic region. The extracellular region of TF has two fibronectin III-like domains and a distribution of disulfide bridges characteristic of class-II cytokine and interferon receptors. The cytoplasmic region of TF is short but contains at least one serine residue that can be phosphorylated.

[0003] Tissue factor forms a tight complex ($K_d \sim$ pmol) with its native ligand - factor VIIa. In the complex, VIIa wraps around tissue factor (Banner, D.W., *et al., Nature 380*:41-46 (1996)) and forms an extensive region of contact with the tissue factor surface.

[0004] Patients with cancer exhibit a much higher than expected incidence of thromboembolic disorders, commonly referred to as Trousseau syndrome. Many tumor types commonly associated with Trousseau syndrome, such as lung, pancreatic, breast, colon, and gastric carcinomas, stain positively for TF (Hu, T., *et al., Oncol. Res. 6*:321-327 (1994); Callander, N.S., *et al., Cancer 70*:1194-201 (1992)). Abnormally high expression of TF has been shown clinically to be associated with poor differentiation of many tumors, including colorectal carcinoma (Shigemori, C., *et al., Thromb. Haemost. 80*:894-898 (1998); Seto, S., *et al., Cancer 88*:295-301 (2000)); and non-small cell lung

cancer (Sawada, M., *et al., Br. J. Cancer 79*:472-477 (1999)). Molecular analysis of gene expression shows that TF is differentially expressed in breast cancer cells (Kirschmann, D.A., *et al., Breast Cancer Res. Treat. 55*:127-136 (1999); Schwirzke, M., *et al., Anticancer Res. 19*:1801-1814 (1999)).

[0005] In tumor tissues, TF is not only expressed on the surface of tumor cells but also on tumor associated vascular endothelial cells. TF has been shown to play an essential role in embryonic blood vessel development (Carmeliet, P., *et al., Nature 383*:73-75 (1996)). TF normally is not expressed in the endothelium. However, tumor associated vascular endothelial cells in breast cancer (Contrino, J., *et al., Nat. Med. 2*:209-215 (1996); Shoji, M., *et al., Am. J. Pathol. 152*:399-411 (1998)), pituitary adenoma (Nishi, T., *et al., Cancer 86*:1354-1361 (1999)) and lung cancer (Shoji, M., *et al., Am. J. Pathol. 152*:399-411 (1998); Koomagi, R. and Volm, M., *Int. J. Cancer 79*:19-22 (1998)) have been shown to express TF. The expression of TF by tumor cells and tumor associated vascular endothelial cells was shown to be induced by tumor secreted VEGF and TNF (Bierhaus, A., *et al., J. Biol. Chem. 270*:26419-26432 (1995); Zucker, S., *et al., Int. J. Cancer 75*:780-786 (1998); Shen, B.Q., *et al., J. Biol. Chem. 276*:5281-5286 (2001)).

[0006] In normal tissue, TF is only expressed in cells separated from blood proteins by tight endothelium and tissue barriers such as skin, and TF is normally not readily accessible to blood proteins and antibodies. However, in tumor tissues, TF of tumor associated vascular endothelial cells is exposed to blood proteins. At the same time, tumor TF is also accessible because of the leaky tumor vasculature. Tumor cells secrete matrix metalloproteases that most likely play a role in the invasion process, and may be a cause of the leakiness.

[0007] Antibodies that bind to the TF-VIIa interaction site can inhibit TF-VIIa interaction, thus inhibiting or blocking blood coagulation. However, when large quantities of those antibodies are used for tumor therapy, effective bleeding control in patients may be compromised.

SUMMARY OF THE INVENTION

[0008] The present invention is directed to an isolated antibody capable of binding to human tissue factor (hTF), wherein the antibody does not inhibit tissue factor (TF) mediated blood coagulation when compared to a normal plasma control. The present invention is also directed to an isolated antibody capable of binding to hTF, wherein the antibody does not inhibit TF mediated blood coagulation when compared to a normal plasma control, and wherein the antibody can initiate an Fc-mediated mechanism. The antibody can be a monoclonal antibody, a chimeric antibody, a single chain antibody, a humanized antibody, a modified antibody, a heavy or light chain variable region thereof, or an antibody product of a Fab expression library. The present invention is further directed to hybridomas producing such an antibody, as well as nucleic acid molecules encoding such an antibody.

[0009] The present invention is further directed to an immunoglobulin molecule comprising the heavy or light chain variable region of the antibody.

[0010] The present invention is further directed to an anti-antibody capable of interfering with the binding of the antibody to hTF.

[0011] The present invention is further directed to a method of producing a monoclonal antibody of the invention, the method comprising: (a) immunizing a mammal with a polypeptide comprising a purified extracellular domain of hTF; (b) preparing a cell suspension from lymph nodes of the immunized mammal; (c) fusing cells from the cell suspension of step (b) with myeloma cells; and (d) identifying a clone from a hybridoma generated from the fusion in (c), wherein the clone produces an antibody capable of binding to hTF but does not inhibit TF mediated blood coagulation compared to a normal plasma control, and optionally wherein the antibody can initiate an Fc-mediated mechanism.

[0012] The invention is further directed to a pharmaceutical composition comprising a therapeutically effective amount of the antibody of the invention and a pharmaceutically acceptable carrier.

[0013] The present invention is also directed to a method of treating cancer in a patient, the method comprising administering to the patient a therapeutically effective amount of the antibody of the invention. The antibody can be conjugated to a cytotoxic agent or a radionuclide.

[0014] The invention is further directed to an isolated polynucleotide having a nucleotide sequence encoding an antibody of the invention. The invention is further directed to an isolated polynucleotide having a nucleotide sequence encoding a polypeptide that is at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical in amino acid sequence to an antibody, or antibody fragment thereof, of the invention. In some embodiments, such a polypeptide has the immunospecificity of an antibody of the present invention. The invention is also directed to a vector comprising the isolated polynucleotide, and a host cell comprising the vector.

[0015] The invention is further directed to a kit comprising a pharmaceutical composition comprising a therapeutically effective amount of the antibody of the invention. In some embodiments, the kit further comprises printed matter which provides information on the use of the pharmaceutical composition to treat cancer or a pre-recorded media device which provides information on the use of the pharmaceutical composition to treat cancer or a planner.

[0016] The present invention is also directed to a method of delivering a pharmaceutical composition comprising a therapeutically effective amount of the antibody of the present invention to a patient in need thereof, the method comprising (a) registering in a computer readable medium the identity of a physician permitted to prescribe the pharmaceutical composition; (b) providing the patient with counseling information concerning the risks attendant to the pharmaceutical composition; (c) obtaining informed consent from the patient to receive the pharmaceutical composition despite the attendant risks; (d) registering the patient in a computer readable medium after obtaining their informed consent; and (e) permitting the patient access to the pharmaceutical composition.

[0017] The present invention is also directed to a method of educating consumers about the use of a pharmaceutical composition, the method comprising distributing the pharmaceutical composition with consumer information at a point of sale.

[0018] The present invention is also directed to a method of detecting cancer, the method comprising providing the antibody of the invention, conjugated to a detectable agent, to a sample or subject and detecting the detectable agent bound to cancer cells.

[0019] The present invention is further directed to a method of identifying a pharmaceutical composition comprising a therapeutically effective amount of the antibody of the invention, and commercializing the same as a drug, the method comprising (a) isolating an antibody capable of binding to human tissue factor, wherein the antibody does not inhibit tissue factor mediated blood coagulation compared to normal plasma controls and can initiate an Fc-mediated mechanism; (b) repeating (a) to obtain a plurality of candidate antibodies that may prove therapeutically effective; (c) demonstrating that one such candidate antibody is non-toxic when administered to a non-human animal; (d) conducting a supervised clinical trial to demonstrate the non-toxic and effective character of one such candidate antibody; (e) securing approval of a regulatory agency to distribute one such candidate antibody to treat cancer; and (f) making a pharmaceutical composition comprising the candidate antibody as the active agent.

BRIEF DESCRIPTION OF THE FIGURES

[0020] FIG. 1. Purification of soluble human tissue factor (hTF) using Ni-agarose column. Lane 1 contains molecular weight markers. Lane 2 is pre-purification and lanes 3-4 are flow-through and wash samples. Lanes 5-9 are fractions eluted with an imidazole gradient. The arrow indicates soluble hTF. Fractions 11-14 that contained protein bands as in lane 8 were pooled and further purified using gel-filtration chromatography. Fractions 15-26 that contained protein bands as in lane 9 were pooled and used for immunization.

[0021] FIG. 2. Purification of hTF using gel-filtration chromatography. Lane 1 contains molecular weight markers. Lanes 2-12 are fractions eluted from the gel filtration column. Fractions with protein bands as in lanes 6 to 12 were pooled. Both bands (arrow indicated) are soluble hTF as demonstrated by Western-blot analysis.

[0022] FIGS. 3A-3C. FACS analysis of selected hTF stable cell clones. FACS analysis was carried out with a commercial anti-TF antibody (10 μg/ml, Calbiochem, CA, Cat. #612161) as the first antibody and a FITC-labeled goat anti-mouse IgG (1:50 dilution, Southern Biotechnology, AL) as the second antibody. The fluorescence intensities of the antibody stained cells were measured on a flow cytometer (FACScan, Becton Dickinson, NJ) and analyzed using Cell Quest software (Becton Dickinson, NJ). Fig. 3A. Untransfected CHO cells. Fig. 3B. Representative stable clone #TF34. Fig. 3C. Representative stable clone #TF48.

[0023] FIGS. 4A-4C. ADCC assays using human chimeric anti-TF antibodies TF260, TF278 and TF392. TF-positive SW900 and TF-negative A549 lung tumor cells were used as targets. An irrelevant human IgG1 was used as the negative control antibody.

[0024] FIG. 5. Nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of full length human tissue factor with a 32 amino acid N-terminal leader sequence and a 9 amino acid C-terminal RGS-His_6 tag sequence.

[0025] FIG. 6. Nucleotide (SEQ ID NO:3) and amino acid (SEQ ID NO:4) sequences of the extracellular domain of human tissue factor with a 32 amino acid N-terminal leader sequence and a 9 amino acid C-terminal RGS-His_6 tag sequence.

[0026] FIGS. 7A-7B. Antibody TF260. FIG. 7A. Nucleotide (SEQ ID NO:5) and amino acid (SEQ ID NO:6) sequences of TF260 VH (TF260VH/PUC18). FIG. 7B. Nucleotide (SEQ ID NO:7) and amino acid (SEQ ID NO:8) sequences of TF260 VL (TF260VL/PUC18).

[0027] FIGS. 8A-8B. Antibody TF196. FIG. 8A. Nucleotide (SEQ ID NO:9) and amino acid (SEQ ID NO:10) sequences of TF196 VH

(TF196VH/PUC18). FIG. 8B. Nucleotide (SEQ ID NO:11) and amino acid (SEQ ID NO:12) sequences of TF196 VL (TF196VH/PUC18).

[0028] FIGS. 9A-9B. Antibody TF278. FIG. 9A. Nucleotide (SEQ ID NO:18) and amino acid (SEQ ID NO:19) sequences of TF278 VH (TF278VHs-PUC18). FIG. 9B. Nucleotide (SEQ ID NO:20) and amino acid (SEQ ID NO:21) sequences of TF278 VL (TF278VLs-PUC18).

[0029] FIGS. 10A-10B. Antibody TF277. FIG. 10A. Nucleotide (SEQ ID NO:22) and amino acid (SEQ ID NO:23) sequences of TF277 VH. FIG. 10B. Nucleotide (SEQ ID NO:24) and amino acid (SEQ ID NO:25) sequences of TF277 VL.

[0030] FIG. 11. Antibody TF392. Nucleotide (SEQ ID NO:26) and amino acid (SEQ ID NO:27) sequences of TF392 VH (TF392VHs-PUC18). The nucleotide and amino acid sequences of TF392 VL are the same as the nucleotide (SEQ ID NO:7) and amino acid (SEQ ID NO:8) sequences of TF260 VL.

[0031] FIGS. 12A-12B. Antibody TF9. FIG. 12A. Nucleotide (SEQ ID NO:28) and amino acid (SEQ ID NO:29) sequences of TF9VH (TF9VHs-PUC18). FIG. 12B. Nucleotide (SEQ ID NO:30) and amino acid (SEQ ID NO:31) sequences of TF9 VL (TF9VL-PUC18).

[0032] Note: In FIGS. 7A-12B, the underlined amino acid residues specify the CDR regions of the VH or VL region of antibodies TF260, TF196, TF278, TF277, TF392 and TF9, respectively.

[0033] Note: A 57 nucleotide (a 19 amino acid) sequence signal peptide:

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ATG GCT TGG GTG TGG ACC TTG CTA TTC CTG ATG GCA GCT
 M   A   W   V   W   T   L   L   F   L   M   A   A

GCC CAA AGT GCC CAA GCA (SEQ ID NO:32)
 A   Q   S   A   Q   A  (SEQ ID NO:33)
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was used in constructing a vector containing VH and VL of TF260, TF196, TF278, TF277, TF392, or VH of TF9. A 60 nucleotide (a 20 amino acid) sequence signal peptide:

ATG GAA TCA CAG ACT CAG GTC TTC CTC TCC CTG CTG CTC
M E S Q T Q V F L S L L L

TGG ATA TCT GGT ACC TGT GGG (SEQ ID NO:34)
W I S G T C G (SEQ ID NO:35)

was used in constructing a vector containing VL of TF 9.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0034] In normal tissue, TF is only expressed in cells separated from blood proteins by tight endothelium and tissue barriers such as skin. TF is normally not accessible to blood proteins including antibodies, since TF is not usually expressed on the surface of cells that are in direct contact with blood such as the endothelium lining vessels. However, TF is expressed by many types of tumor cells including tumor associated vascular endothelial cells, where TF is exposed to blood proteins. TF is involved in embryonic blood vessel development and has been associated with tumor metastasis. Thus, TF is considered to be a potential tumor therapeutic target.

Antibodies

[0035] The invention is directed to an isolated antibody capable of binding to human TF (hTF), wherein the antibody does not inhibit TF mediated blood coagulation compared to a normal plasma control. The invention is also directed to an isolated antibody capable of binding to hTF, wherein the antibody does not inhibit TF mediated blood coagulation compared to a normal plasma control and wherein the antibody can initiate one or more Fc-mediated mechanisms. Because the antibodies of the invention do not inhibit normal TF mediated blood coagulation, normal plasma clotting is not affected in patients treated with antibodies of the invention.

[0036] As used herein, "isolated" refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is altered "by the hand of man" from its natural state.

[0037] The basic antibody structural unit is known to comprise a tetramer composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxyl-terminal portion of each chain defines a constant region primarily responsible for Fc-mediated mechanisms. Human light chains are classified as kappa and lambda light chains. Heavy chains are classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. See generally, *Fundamental Immunology*, Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). The variable regions of each light/heavy chain pair form the antigen binding site. Thus, an intact IgG antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are the same.

[0038] The variable regions all exhibit the same general structure of relatively conserved framework regions (FR) joined together by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the heavy and the light chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N-terminal to C-terminal, both variable region light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987 and 1991)); Chothia & Lesk, *J. Mol. Biol. 196*:901-917 (1987); or Chothia *et al., Nature 342*:878-883 (1989).

[0039] As used herein, the term "antibody" is intended to refer to intact immunoglobulin molecules and immunologically active portions or fragments of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. The antibody of the invention is capable of specifically binding to hTF, without inhibiting TF mediated blood coagulation compared to a normal plasma control. In certain embodiments, the antibody of the invention is capable of specifically binding

to hTF, without inhibiting TF mediated blood coagulation compared to a normal plasma control, and wherein the antibody can initiate one or more Fc-mediated mechanisms

[0040] Antibodies of the invention include, but are not limited to, intact monoclonal, multispecific, human, humanized and chimeric antibodies, modified antibodies, single chain antibodies, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), Fab fragments, F(ab') fragments, $F(ab')_2$ fragments, Fv fragments, fragments produced by a Fab expression library, fragments comprising either a VL or VH domain, anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), intracellularly-made antibodies (i.e., intrabodies), and antigen-binding antibody fragments. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. In some embodiments, the immunoglobulin is an IgG1 isotype. In other embodiments, the immunoglobulin is an IgG2 isotype. In yet other embodiments, the immunoglobulin is an IgG4 isotype. Immunoglobulins can have both heavy and light chains. An array of IgG, IgE, IgM, IgD, IgA, and IgY heavy chains can be paired with a light chain of the kappa or lambda form.

[0041] Antibodies of the invention can also comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, CH3 and/or Fc domain(s). The antibodies of the invention can be from any animal origin, including birds and mammals. In some embodiments, the antibodies are human, murine, rat, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins. Thus, it should be understood that antibodies of the invention from an animal are capable of binding to human tissue factor and do

not inhibit TF mediated blood coagulation when compared to a normal plasma control. Such antibodies can initiate one or more Fc-mediated mechanisms.

[0042] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that can be present in minor amounts. In contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen and is therefore highly specific. In addition to their specificity, monoclonal antibodies are advantageous since they are synthesized by hybridoma cultures, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention can be made by the hybridoma method first described by Kohler & Milstein, *Nature 256*:495 (1975), or alternatively can be made by recombinant DNA methods (see e.g., U.S. Patent No. 4,816,567 (Cabilly *et al.*)).

[0043] "Chimeric" antibodies (immunoglobulins) refer to those antibodies having a portion of the heavy and/or light chain which is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (*see* U.S. Patent No. 4,816,567 (Cabilly *et al.*); Morrison *et al., Proc. Nat. Acad. Sci. USA 81*:6851-6855 (1984)).

[0044] "Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding sub-sequences of antibodies)

which contain minimal sequence derived from a non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity and affinity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, a humanized antibody can comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin, and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, *see*, Jones *et al., Nature 321*:522-525 (1986); Reichmann *et al., Nature 332*:323-329 (1988); and Presta, *Curr. Op. Struct. Biol. 2*:593-596 (1992).

[0045] The term "modified antibody" refers to an antibody that has been modified with respect to effector function, so as to enhance the effectiveness of the antibody at mediating antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) (also known as complement-mediated cell killing). For example, cysteine residue(s) can be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated can have improved internalization capability and/or increased ADCC and CDC. See Caron *et al., J. Exp. Med. 176*:1191-1195 (1992) and Shopes, B., *J. Immunol. 148*: 2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity can also be prepared using heterobifunctional cross-linkers as described in Wolff *et al., Cancer Research 53*: 2560-2565 (1993). Alternatively, an antibody can be modified such that it has dual Fc regions and

can thereby have enhanced complement-mediated lysis and ADCC capabilities. See Stevenson *et al., Anti-Cancer Drug Design 3*:219-230 (1989). Additionally, an antibody can be engineered to produce glycoforms which have altered glycosylation patterns that result in enhanced ADCC activity. See U.S. Patent No. 6,602,684.

[0046] A bispecific or bifunctional antibody is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of F(ab') fragments. See, e.g., Songsivilai & Lachmann, *Clin. Exp. Immunol. 79*:315-321 (1990), Kostelny *et al., J. Immunol. 148*:1547 1553 (1992). In addition, bispecific antibodies can be formed as "diabodies" (Holliger *et al., PNAS USA 90*:6444-6448 (1993)) or "Janusins" (Traunecker *et al., EMBO J. 10*:3655-3659 (1991) and Traunecker *et al., Int. J. Cancer Suppl. 7*:51-52 (1992)).

[0047] The invention is also directed to an immunoglobulin molecule comprising the heavy or light chain variable region of the antibody of the invention. The invention is further directed to an isolated anti-antibody capable of interfering with the binding of the antibody of the invention to hTF, wherein said anti-antibody does not inhibit TF mediated blood coagulation compared to a normal plasma control.

[0048] Antibodies of the present invention can also be described or specified in terms of their cross-reactivity. In some embodiments, antibodies of the invention bind polypeptides having at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, at least about 50%, at least about 45%, or at least about 40% amino acid sequence identity to a TF polypeptide (e.g., human TF (SEQ ID NO:2)), or a fragment of the TF polypeptide. In some embodiments, antibodies of the present invention cross-react with murine, monkey, rat and/or rabbit homologs of hTF and the corresponding epitopes thereof. In other embodiments, the above-described cross-reactivity is with respect to any single specific antigenic or

immunogenic polypeptide, or combination(s) of 2, 3, 4, 5, or more of specific antigenic and/or immunogenic polypeptides.

[0049] As known in the art, "sequence identity" between two polypeptides is determined by comparing the amino acid sequence of one polypeptide to the sequence of a second polypeptide. When discussed herein, whether any particular polypeptide is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% identical to another polypeptide can be determined using methods and computer programs/software known in the art such as, but not limited to, the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). BESTFIT uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics 2*:482-489 (1981), to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for example, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference polypeptide sequence and that gaps in homology of up to 5% of the total number of amino acids in the reference sequence are allowed.

[0050] The antibodies of the invention can bind immunospecifically to a hTF polypeptide or a polypeptide fragment of hTF. In some embodiments, the antibodies of the invention bind immunospecifically to hTF. In other embodiments, the antibodies of the invention bind immunospecifically to the extracellular domain of hTF. As used herein, "extracellular domain of hTF" is intended to refer to the 219 amino acid residue portion of hTF that is localized on the outside surface of the cell (see e.g., Fig. 6, providing the nucleotide (SEQ ID NO:3) and amino acid (SEQ ID NO:4) sequences of the extracellular domain of human tissue factor with a 32 amino acid N-terminal leader sequence and a 9 amino acid C-terminus RGS-His_6 tag sequence).

[0051] In some embodiments, the antibodies of the present invention preferentially bind to hTF. In other embodiments, the antibodies of the

present invention immunospecifically bind to hTF and do not cross-react with any other antigens. The antibodies of the present invention do not inhibit TF mediated blood coagulation compared to a normal plasma control. In other embodiments, the antibodies of the invention initiate one or more Fc-mediated mechanisms.

[0052] The term "antigen-binding antibody fragment" is intended to refer to a molecule (e.g., a polypeptide) which is a portion or part of a polypeptide sequence as compared to a corresponding full length or native polypeptide sequence of an antibody of the invention. The portion or part of a polypeptide sequence can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or at least about 99% of the full length or native polypeptide sequence of a full size antibody of the invention, but which retains at least some degree of binding specificity of the full length antibody, and does not inhibit TF mediated blood coagulation compared to a normal plasma control, and optionally, initiate an Fc-mediated mechanism.

[0053] Antigen-binding antibody fragments (including derivatives) of the antibody molecules (e.g., the VH domains and/or VL domains) described herein include, but are not limited to, fragments (including derivatives) of at least 20, at least 40, at least 60, at least 80, at least 100, at least 120, at least 140, at least 160, or more than 160 amino acids of the full length antibody, including the VH domain, VHCDR1, VHCDR2, VHCDR3, VL domain, VLCDR1, VLCDR2, or VLCDR3. The resultant antibody or antigen-binding antibody fragments can be screened for biological activity to identify fragments that retain the desired activity (e.g., the ability to bind hTF).

[0054] By way of a non-limiting example, an antibody can be considered to bind hTF preferentially if it binds the protein with a dissociation constant (K_D) or an off rate (K_{off}), that is less than the antibody's K_D or K_{off} for a second antigen. In other non-limiting embodiments, an antibody can be considered to bind hTF preferentially if it binds the protein with a K_D or K_{off} that is at least one order of magnitude less than the antibody's K_D or K_{off} for the second

antigen. In other non-limiting embodiments, an antibody can be considered to bind hTF preferentially if it binds hTF with a K_D or K_{off} that is at least two orders of magnitude less than the antibody's K_D or K_{off} for the second antigen.

[0055] Antibodies of the present invention can also be described or specified in terms of their binding affinity to hTF. In some embodiments, binding affinities include those with a dissociation constant or K_D less than or equal to 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, or 10^{-4} M. In other embodiments, affinities include those with a dissociation constant or K_D less than or equal to 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6}M, 5×10^{-7} M, 10^{7} M, 5×10^{-8} M or 10^{-8} M. In yet other embodiments, binding affinities include those with a dissociation constant or K_D less than or equal to 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

[0056] In some embodiments, antibodies of the invention can bind hTF polypeptides with an off rate (K_{off}) of less than or equal to 5×10^{-2} sec^{-1}, 10^{-2} sec^{-1}, 5×10^{-3} sec^{-1} or 10^{-3} sec^{-1}. In other embodiments, antibodies of the invention can bind hTF polypeptides or fragments thereof with an off rate (K_{off}) less than or equal to 5×10^{-4} sec^{-1}, 10^{-4} sec^{-1}, 5×10^{-5} sec^{-1}, or 10^{-5} sec^{-1} 5×10^{-6} sec^{-1}, 10^{-6} sec^{-1}, 5×10^{-7} sec^{-1} or 10^{-7} sec^{-1}.

[0057] In some embodiments of the present invention, antibodies that immunospecifically bind to hTF can comprise a polypeptide having the amino acid sequence of any one of the heavy chains expressed by an anti-TF antibody expressing cell line of the invention and/or any one of the light chains expressed by an anti-TF antibody expressing cell line of the invention. In other embodiments of the present invention, antibodies that immunospecifically bind to hTF can comprise a polypeptide having the amino acid sequence of any one of the VH domains of a heavy chain expressed by an anti-TF antibody expressing cell line of the invention and/or any one of the VL domains of a light chain expressed by an anti-TF antibody expressing cell line of the invention. In yet other embodiments, antibodies of the present invention can comprise the amino acid sequence of a VH domain and VL domain expressed by a single anti-TF antibody expressing cell line of the

invention. In other embodiments, antibodies of the present invention can comprise the amino acid sequence of a VH domain and a VL domain expressed by two different anti-TF antibody expressing cell lines of the invention. Molecules comprising, or alternatively consisting of, antigen-binding antibody fragments of the VH and/or VL domains expressed by an anti-TF antibody expressing cell line of the invention that immunospecifically bind to hTF are also encompassed by the invention, as are nucleic acid molecules encoding these VH and VL domains, molecules, and/or fragments.

[0058] The present invention also provides polypeptides that comprise, or alternatively consist of, variants (including derivatives) of the antibody molecules (e.g., the VH domains and/or VL domains) described herein, which polypeptides immunospecifically bind to hTF or a fragment or variant thereof. The term "variant" refers to a molecule (e.g., a polypeptide or polynucleotide sequence) with at least one or more differences in its amino acid or nucleotide sequence as compared to a corresponding native polypeptide or DNA sequence. Amino acid sequence variants of the invention will possess at least about 70%, at least about 75%, at least about 85%, at least about 95%, or at least about 99% sequence identity with the amino acid sequence of an anti-TF antibody of the invention. Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions can be single, where only one amino acid in the molecule has been substituted, or they can be multiple, where two or more amino acids have been substituted in the same molecule. Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native amino acid sequence connected to either the α-carboxyl or α-amino functional group of the amino acid. Deletional variants are those with one or more amino acids removed from the native amino acid sequence. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule. Standard techniques known to those of skill in the art can be used to introduce mutations into an antibody of the invention, including, for example, by site-directed mutagenesis or PCR-mediated mutagenesis of the

encoding nucleic acid molecule which results in amino acid substitutions. In some embodiments, the variant (including derivatives) has less than 50 amino acid substitutions, less than 40 amino acid substitutions, less than 30 amino acid substitutions, less than 25 amino acid substitutions, less than 20 amino acid substitutions, less than 15 amino acid substitutions, less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid substitutions relative to the reference polypeptide. In some embodiments, the variant polypeptide has the same immunospecificity, or binds the same epitope, as a polypeptide of the present invention.

[0059] It is well known in the art that polypeptides, or fragments or variants thereof, with similar amino acid sequences can have similar structure and many of the same biological activities. Thus, the invention is further directed to an isolated first antibody, or antigen-binding fragment thereof, having an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a second antibody comprising an amino acid sequence selected from the group consisting of: (a) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF196 (deposited May 15, 2003, ATCC Deposit No. PTA-5196); (b) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF260 (deposited May 15, 2003, ATCC Deposit No. PTA-5197); (c) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF278 (deposited December 3, 2003, ATCC Deposit No. PTA-5676); (d) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF277 (deposited December 3, 2003, ATCC Deposit No. PTA-5675); (e) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF392 (deposited December 3, 2003, ATCC Deposit No. PTA-5677); (f) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF9 (deposited December 3, 2003, ATCC Deposit No. PTA-5674); (g) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF196; (h) at least two CDR regions of a VH

domain of the antibody expressed by hybridoma cell line TF260; (i) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF278: (j) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF277; (k) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF392; (l) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF9; (m) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF196; (n) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF260; (o) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF278: (p) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF277; (q) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF392; (r) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF9; (s) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF196; (t) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF260; (u) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF278; (v) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF277; (w) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF392; (x) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF9; (y) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF196; (z) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF260; (aa) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF278; (bb) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF277; (cc) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF392; (dd) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF9; (ee) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line

TF196; (ff) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF260; (gg) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF278; (hh) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF277; (ii) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF392; and (jj) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF9. In some embodiments, the first antibody, or antigen-binding fragment thereof, has the same immunospecificity, or binds the same epitope, as the second antibody.

[0060] The invention is directed to an isolated antibody, or antigen-binding fragment thereof, having an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, or at least 95% identical to an amino acid sequence selected from the group consisting of: (a) a polypeptide of SEQ ID NO:6 or 8 or a polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5252 or PTA-5253, respectively (TF260VH/PUC18 or TF260VL/PUC18, respectively); (b) a polypeptide domain of SEQ ID NO:6 or 8 or the polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5252 or PTA-5253, respectively (TF260VH/PUC18 or TF260VL/PUC18, respectively); (c) a polypeptide epitope of SEQ ID NO:6 or 8 or the polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5252 or PTA-5253, respectively (TF260VH/PUC18 or TF260VL/PUC18, respectively); (d) a polypeptide which is a variant of SEQ ID NO:6 or 8; (e) a polypeptide which is a species homologue of the SEQ ID NO:6 or 8; (f) a polypeptide of SEQ ID NO:10 or 12 or a polypeptide encoded by the cDNA sequence included in ATCC Deposit No: PTA-5250 or PTA-5251, respectively (TF196VH/PUC18 or TF196VL/PUC18, respectively); (g) a polypeptide domain of SEQ ID NO:10 or 12 or the polypeptide encoded by the cDNA sequence included in ATCC Deposit No: PTA-5250 or PTA-5251, respectively (TF196VH/PUC18 or TF196VL/PUC18, respectively); (h) a polypeptide epitope of SEQ ID NO:10 or 12 or the polypeptide encoded by the cDNA sequence included in ATCC Deposit No: PTA-5250 or PTA-5251,

respectively (TF196VH/PUC18 or TF196VL/PUC18, respectively); (i) a polypeptide which is a variant of SEQ ID NO:10 or 12; (j) a polypeptide which is a species homologue of SEQ ID NO:10 or 12; (k) a polypeptide of SEQ ID NO:19 or 21 or a polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5694 or PTA-5695, respectively (TF278VHs/PUC18 OR TF278VLs/PUC18, respectively); (l) a polypeptide domain of SEQ ID NO:19 or 21 or the polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5694 or PTA-5695, respectively (TF278VHs/PUC18 OR TF278VLs/PUC18, respectively); (m) a polypeptide epitope of SEQ ID NO:19 or 21 or the polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5694 or PTA-5695, respectively (TF278VHs/PUC18 OR TF278VLs/PUC18, respectively); (n) a polypeptide which is a variant of SEQ ID NO:19 or 21; (o) a polypeptide which is a species homologue of the SEQ ID NO:19 or 21; (p) a polypeptide of SEQ ID NO:23 or 25; (q) a polypeptide domain of SEQ ID NO:23 or 25; (r) a polypeptide epitope of SEQ ID NO:23 or 25; (s) a polypeptide which is a variant of SEQ ID NO:23 or 25; (t) a polypeptide which is a species homologue of the SEQ ID NO:23 or 25; (u) a polypeptide of SEQ ID NO:27 or a polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5696 (TF392VHs/PUC18); (v) a polypeptide domain of SEQ ID NO:27 or the polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5696 (TF392VHs/PUC18); (w) a polypeptide epitope of SEQ ID NO:27 or the polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5696 (TF392VHs/PUC18); (x) a polypeptide which is a variant of SEQ ID NO:27; (y) a polypeptide which is a species homologue of the SEQ ID NO:27; (z) a polypeptide of SEQ ID NO:29 or 31 or a polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5692 or PTA-5693, respectively (TF9VHs/PUC18 or TF9VL/PUC18, respectively); (aa) a polypeptide domain of SEQ ID NO:29 or 31 or the polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5692 or PTA-5693, respectively (TF9VHs/PUC18 or TF9VL/PUC18, respectively); (bb) a polypeptide epitope of SEQ ID NO:29 or 31 or the

polypeptide encoded by the cDNA sequence provided in ATCC Deposit No: PTA-5692 or PTA-5693, respectively (TF9VHs/PUC18 or TF9VL/PUC18, respectively); (cc) a polypeptide which is a variant of SEQ ID NO:29 or 31; and (dd) a polypeptide which is a species homologue of the SEQ ID NO:29 or 31. In some embodiments, the antibody, or antigen-binding fragment thereof, has the same immunospecificity, or binds the same epitope, as the polypeptide encoded by the amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 8, 10, 12, 19, 21, 23, 25, 27, 29 and 31.

[0061] The invention also encompasses antibodies that have one or more of the same biological characteristics as one or more of the antibodies described herein. By "biological characteristics" is intended to mean the *in vitro* or *in vivo* activities or properties of the antibodies, such as, for example, the ability to bind to TF (e.g., hTF expressed on a cell surface, or membrane-embedded hTF), without inhibiting TF mediated blood coagulation compared to a normal plasma control. Optionally, the antibodies of the invention can bind to the same epitope as one of the antibodies specifically referred to herein. Such epitope binding can be routinely determined using assays known in the art.

[0062] The invention is also directed to a monoclonal antibody having the binding characteristics of, or that competes for binding to an epitope recognized by, a monoclonal antibody produced by hybridoma cell line TF260. The invention is also directed to a monoclonal antibody having the binding characteristics of, or that competes for binding to an epitope recognized by, a monoclonal antibody produced by hybridoma cell line TF196. The invention is also directed to a monoclonal antibody having the binding characteristics of, or that competes for binding to an epitope recognized by, a monoclonal antibody produced by hybridoma cell line TF278. The invention is also directed to a monoclonal antibody having the binding characteristics of, or that competes for binding to an epitope recognized by, a monoclonal antibody produced by hybridoma cell line TF277. The invention is also directed to a monoclonal antibody having the binding characteristics of, or that competes for binding to an epitope recognized by, a monoclonal antibody produced by hybridoma cell line

TF392. The invention is also directed to a monoclonal antibody having the binding characteristics of, or that competes for binding to an epitope recognized by, a monoclonal antibody produced by hybridoma cell line TF9. The invention is also directed to an antibody obtainable from a hybridoma cell line TF260. The invention is also directed to an antibody obtainable from a hybridoma cell line TF196. The invention is also directed to an antibody obtainable from a hybridoma cell line TF278. The invention is also directed to an antibody obtainable from a hybridoma cell line TF277. The invention is also directed to an antibody obtainable from a hybridoma cell line TF392. The invention is also directed to an antibody obtainable from a hybridoma cell line TF9. The invention is also directed to a hybridoma cell line which produces antibodies which have the same immunospecificity, or bind the same epitope, as antibodies produced from hybridoma cell line TF260. The invention is also directed to a hybridoma cell line which produces antibodies which have the same immunospecificity, or bind the same epitope, as antibodies produced from hybridoma cell line TF196. The invention is also directed to a hybridoma cell line which produces antibodies which have the same immunospecificity, or bind the same epitope, as antibodies produced from hybridoma cell line TF278. The invention is also directed to a hybridoma cell line which produces antibodies which have the same immunospecificity, or bind the same epitope, as antibodies produced from hybridoma cell line TF277. The invention is also directed to a hybridoma cell line which produces antibodies which have the same immunospecificity, or bind the same epitope, as antibodies produced from hybridoma cell line TF392. The invention is also directed to a hybridoma cell line which produces antibodies which have the same immunospecificity, or bind the same epitope, as antibodies produced from hybridoma cell line TF9. The invention is also directed to hybridoma cell line TF260. The invention is also directed to hybridoma cell line TF196. The invention is also directed to hybridoma cell line TF278. The invention is also directed to hybridoma cell line TF277. The invention is also directed to hybridoma cell line TF392. The invention is also directed to hybridoma cell line TF9. The invention is also directed to an antibody comprising an amino

acid sequence of SEQ ID NO:6, 8, 10, 12, 19, 21, 23, 25, 27, 29, or 31. The American Type Culture Collection (ATCC) is located at 10801 University Boulevard, Manassas, VA 20110-2209, USA.

Methods of Producing Antibodies

[0063] The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, e.g., by chemical synthesis or by recombinant expression techniques. In other embodiments, rapid immunization of mice at multiple sites (RIMMS) can be used. See, e.g., Kilpatrick, K.E., *et al.*, *Hybridoma* 16:381-389 (1997). In yet other embodiments, methods of producing antibodies include, but are not limited to, hybridoma technology, EBV transformation, XenoMouse™ technology (*see* Green *et al.*, *Nature Genetics* 7:13-21 (1994) and other methods discussed herein as well as through the use of recombinant DNA technology, as discussed below.

[0064] The antibodies of the present invention can be generated by any suitable method known in the art. Polyclonal antibodies to an antigen of interest can be produced by various procedures well known in the art. For example, a polypeptide of interest can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants can be used to increase the immunological response, depending on the host species, and include, but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

[0065] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or combinations thereof. For example,

monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, *et al.*, in *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, New York (1981), pp. 563-681. The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0066] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with a polypeptide of interest or a cell expressing such peptide. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, e.g., mouse myeloma cells (P3X63/Ag8.653, ATCC No. CRL-1580, Manassas, VA; SP2/0-Ag14, ATCC No. CRL-1581, Manassas, VA; P3/NSI/1-Ag4-1 (NS-1), ATCC No. TIB-18, Manassas, VA). Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of interest. Ascites fluid, which generally contains high levels of antibodies, can be generated by injecting mice with positive hybridoma clones.

[0067] Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention, wherein the hybridoma is generated by fusing splenocytes or lymph node cells isolated from a mammal immunized with an antigen of the invention with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of interest.

[0068] Humanized antibodies can be made by a variety of methods known in the art including phage display methods using antibody libraries derived from human immunoglobulin sequences. See also U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741. Additionally, antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology 28*(4/5):489-498 (1991); Studnicka *et al., Protein Engineering 7*(6):805-814 (1994); Roguska., *et al., PNAS 91*:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

[0069] Human antibodies can be produced using transgenic mice that are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy chain and light chain immunoglobulin gene complexes can be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, nucleic acids encoding the human variable region and constant region can be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy chain and light chain immunoglobulin genes can be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In some embodiments, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring that express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of interest. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and

subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, *Int. Rev. Immunol. 13*:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; 5,939,598; 6,075,181; and 6,114,598.

[0070] Completely human antibodies that recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope (Jespers *et al., Bio/technology 12*:899-903 (1988)).

[0071] Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patent Nos. 4,946,778 and 5,258,498; Huston *et al., Methods in Enzymology 203*:46-88 (1991); Shu *et al., PNAS 90*:7995-7999 (1993); and Skerra *et al., Science 240*:1038-1040 (1988). Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in E. coli can also be used (Skerra *et al., Science 242*:1038-1041 (1988)).

[0072] In addition, methods for producing chimeric antibodies are known in the art. See e.g., Morrison, *Science 229*:1202 (1985); Oi *et al., BioTechniques 4*:214 (1986); Gillies *et al., J. Immunol. Methods 125*:191-202 (1989); Neuberger *et al., Nature 312*:604-608 (1984); Takeda *et al., Nature 314*:452-454 (1985); U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397.

[0073] Once an antibody molecule of the invention has been produced by an animal, chemically synthesized, or recombinantly expressed, it can be purified by any method known in the art for purification of an immunoglobulin

molecule, for example, by chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies of the present invention or fragments thereof can be fused to heterologous polypeptide sequences described herein or otherwise known in the art, to facilitate purification.

Nucleic Acid Molecules Encoding Antibodies and Polypeptides Thereof

[0074] The present invention further provides an isolated polynucleotide molecule having a nucleotide sequence encoding an antibody of the invention. In some embodiments, the isolated polynucleotide molecule comprises a nucleotide sequence of SEQ ID NO:5, 7, 9, or 11 (as provided in TF260VH/PUC18, TF260VL/PUC18, TF196VH/PUC18, or TF196VL/PUC18, respectively, deposited June 6, 2003 as ATCC Deposit Nos: PTA-5252, PTA-5253, PTA-5250, or PTA-5251, respectively, or a nucleotide sequence of SEQ ID NO:18, 20, 26, 28, or 30 (as provided in TF278VHs/PUC18, TF278VLs/PUC18, TF392VHs/PUC18, TF9VHs/PUC18 or TF9VL/PUC18, respectively, deposited December 9, 2003 as ATCC Deposit Nos: PTA-5694, PTA-5695, PTA-5696, PTA-5692, or PTA-5693, respectively), or a nucleotide sequence of SEQ ID NO:22 or 24 or a nucleotide sequence encoding an amino acid sequence of SEQ ID NO:6, 8, 10, 12 (as encoded by TF260VH/PUC18, TF260VL/PUC18, TF196VH/PUC18, or TF196VL/PUC18, respectively, deposited June 6, 2003 as ATCC Deposit No: PTA-5252, PTA-5253, PTA-5250, or PTA-5251, respectively), 19, 21, 27, 29, 31 (as encoded by TF278VHs-PUC18, TF278VLs-PUC18, TF392VHs-PUC18, TF9VHs-PUC18 or TF9VL-PUC18, respectively, deposited on December 9, 2003, as ATCC Deposit No: PTA-5694, PTA-5695, PTA-5696, PTA-5692 or PTA-5693, respectively), 23 or 25 (including degenerate variants), or fragments or variants thereof.

[0075] The invention is further directed to an isolated polynucleotide having a nucleotide sequence encoding a first antibody, or antigen-binding fragment

thereof, having an amino acid sequence that is at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the amino acid sequence of a second antibody comprising an amino acid sequence selected from the group consisting of: (a) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF196; (b) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF260; (c) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF278; (d) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF277; (e) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF392; (f) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF9; (g) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF196; (h) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF260; (i) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF278; (j) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF277; (k) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF392; (l) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF9; (m) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF196; (n) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF260; (o) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF278; (p) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF277; (q) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF392; (r) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF9; (s) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF196; (t) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF260; (u) at least one CDR region of a VL domain of the antibody

expressed by hybridoma cell line TF278; (v) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF277; (w) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF392; (x) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF9; (y) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF196; (z) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF260; (aa) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF278; (bb) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF277; (cc) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF392; (dd) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF9; (ee) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF196; (ff) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF260; (gg) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF278; (hh) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF277; (ii) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF392; and (jj) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF9. In some embodiments, the first antibody, or antigen-binding fragment thereof, has the same immunospecificity, or binds the same epitope, as the second antibody.

[0076] The invention is further directed to an isolated polynucleotide having a nucleotide sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a nucleotide sequence encoding the amino acid sequence selected from the group consisting of: (a) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF196; (b) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF260; (c) at least one CDR region of a VH domain of the antibody expressed by

hybridoma cell line TF278; (d) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF277; (e) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF392; (f) at least one CDR region of a VH domain of the antibody expressed by hybridoma cell line TF9; (g) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF196; (h) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF260; (i) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF278; (j) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF277; (k) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF392; (l) at least two CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF9; (m) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF196; (n) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF260; (o) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF278; (p) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF277; (q) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF392; (r) at least three CDR regions of a VH domain of the antibody expressed by hybridoma cell line TF9; (s) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF196; (t) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF260; (u) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF278; (v) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF277; (w) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF392; (x) at least one CDR region of a VL domain of the antibody expressed by hybridoma cell line TF9; (y) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF196; (z) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF260; (aa) at least two CDR regions of a

VL domain of the antibody expressed by hybridoma cell line TF278; (bb) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF277; (cc) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF392; (dd) at least two CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF9; (ee) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF196; (ff) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF260; (gg) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF278; (hh) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF277; (ii) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF392; and (jj) at least three CDR regions of a VL domain of the antibody expressed by hybridoma cell line TF9. In some embodiments, the isolated polynucleotide encodes an amino acid sequence which further encodes an antibody, or antigen-binding fragment thereof, having a CDR region with the same immunospecificity, or binds the same epitope, as either a VH domain or VL domain of an antibody of the present invention.

[0077] The present invention is further directed to a polynucleotide molecule having a nucleotide sequence encoding an antigen-binding antibody fragment that binds to hTF without inhibiting normal TF mediated blood coagulation compared to a normal plasma control, and optionally, can initiate an Fc-mediated mechanism. The present invention is further directed to an isolated polynucleotide molecule having a nucleotide sequence that can hybridize under stringent conditions to the complement of the nucleotide sequence of SEQ ID NO:5, 7, 9, 11, 18, 20, 22, 24, 26, 28, or 30 and that encodes a polypeptide that can bind to hTF without inhibiting normal TF mediated blood coagulation compared to a normal plasma control, and optionally, can initiate an Fc-mediated mechanism. The present invention is further directed to an isolated polynucleotide molecule comprising a nucleotide sequence that has at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to any of SEQ ID NO:5, 7, 9, 11, 18,

20, 22, 24, 26, 28, or 30 and encodes a polypeptide that can bind to hTF without inhibiting normal TF mediated blood coagulation compared to a normal plasma control, and optionally, can initiate an Fc-mediated mechanism.

[0078] As known in the art, "sequence identity" between two nucleotide sequences is determined by comparing the nucleotide sequence of one polynucleotide molecule to the sequence of a second polynucleotide molecule. When discussed herein, whether any particular nucleotide sequence is identical to another nucleotide sequence can be determined using methods and computer programs/software known in the art such as, but not limited to, the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). BESTFIT uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics 2*:482-489 (1981), to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for example, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference polypeptide sequence and that gaps in homology of up to 5% of the total number of nucleic acids in the reference sequence are allowed.

[0079] As used herein "stringent conditions" refer to the ability of a first polynucleotide molecule to hybridize, and remain bound to, a second, filter-bound polynucleotide molecule in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA at 65°C, followed by washing in 0.2 X SSC/0.1% SDS at 42°C (see Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York (1989), at p. 2.10.3).

[0080] The invention is also directed to a recombinant vector comprising an isolated polynucleotide of the present invention, and a host cell comprising the vector. Host cells are genetically engineered (transduced, transformed or

transfected) with a vector of this invention which can be, for example, a cloning vector or an expression vector. The vector can be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, can be those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[0081] The invention is further directed to a method of making an antibody of the invention comprising: (a) expressing the antibody encoded by the isolated polynucleotide of the invention; and (b) recovering the antibody.

[0082] Fragments or portions of the polypeptides of the present invention can be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments can be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention can be used to synthesize full-length polynucleotides of the present invention.

[0083] The polynucleotide molecules of the present invention can be employed for producing polypeptides by recombinant techniques. Thus, for example, a polynucleotide molecule can be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector can be used as long as it is replicable and viable in the host.

[0084] The appropriate DNA sequence can be inserted into a vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) in a vector by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

[0085] The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there can be mentioned: LTR or SV40 promoter, the *E. coli lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also should contain a ribosome binding site for translation initiation, and a transcription terminator. The vector can also include appropriate sequences for amplifying expression, as discussed *infra*.

[0086] In addition, the expression vectors can contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or tetracycline or ampicillin resistance in *E. coli*.

[0087] The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, can be employed to transform an appropriate host cell to permit the host cell to express the protein. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector can be used as long as they are replicable and stable in the host.

[0088] Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retroviruses, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of

ordinary skill in the art. Promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence to be expressed is assembled in appropriate phase with translation initiation and termination sequences, and, if necessary, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal or C-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

[0089] In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by any suitable techniques, such as, e.g., calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation among others. (Davis, L., *et al., Basic Methods in Molecular Biology*, (1986)).

[0090] As representative examples of appropriate hosts, there can be mentioned: bacterial cells, such as *E. coli, Streptomyces, Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein. Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell 23*:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor

and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites can be used to provide the required nontranscribed genetic elements.

[0091] The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

[0092] Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, *et al., Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989).

[0093] Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription or amplifying expression. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

[0094] Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter can be induced by appropriate means (e.g., temperature shift or chemical induction) and the cells are cultured for an additional period.

[0095] Where the desired protein is retained intracellularly, cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or

use of cell lysing agents, or a combination thereof. Such methods are well known to those skilled in the art.

[0096] The polypeptides of the invention can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

[0097] The polypeptides of the present invention can be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptide of the present invention can be glycosylated or can be non-glycosylated. Polypeptides of the invention can also include an initial methionine amino acid residue.

Antibody Conjugates

[0098] Antibodies of the present invention can be used to purify, detect, and/or target hTF, including in both *in vitro* and *in vivo* diagnostic and therapeutic methods. For example, the antibodies can be useful in immunoassays for qualitatively and quantitatively measuring levels of hTF in biological samples. See, e.g., Harlow *et al., Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 2nd ed. 1988).

[0099] The antibodies of the invention include derivatives of antibodies that are modified or conjugated by the covalent attachment of any type of molecule to the antibody. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known

protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivatives can contain one or more non-classical amino acids.

[00100] Antibodies of the present invention can be used for epitope mapping to identify the epitope(s) bound by the antibody. Epitopes identified in this way can, in turn, be used as vaccine candidates, i.e., to immunize an individual to elicit antibodies against the naturally occurring forms of hTF.

[00101] The antibodies of the present invention can be used either alone or in combination with other compositions. The antibodies can be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalent and non-covalent conjugations) to polypeptides or other compositions. For example, antibodies of the present invention can be recombinantly fused or conjugated to molecules useful as labels in detection assays, or as effector molecules such as heterologous polypeptides, drugs, radionuclides, or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 0 396 387.

[00102] In some embodiments, the antibody of the invention is conjugated to cytotoxic agents. A "cytotoxic agent" is any agent toxic or otherwise detrimental to cells. Examples include, but are not limited to, a radionuclide, paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Examples of radionuclides useful as cytotoxic agents include, but are not limited to, ^{131}I, ^{177}Lu, ^{90}Y, and ^{186}Re.

[00103] The present invention also encompasses antibodies of the invention conjugated to detectable agents, wherein the detectable agents can be used for

diagnosis or therapeutic purposes. The antibodies can be used diagnostically to, for example, locate or monitor the development or progression of a tumor as part of a clinical testing procedure to, e.g., determine the efficacy of a given treatment regimen. Examples of detectable agents include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance can be coupled or conjugated either directly to the antibody or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Patent No. 4,741,900 for metal ions that can be conjugated to antibodies for use as diagnostics according to the present invention. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, and acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include iodine (^{121}I, ^{123}I, ^{125}I, ^{131}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{111}In, ^{112}In, ^{113m}In, ^{115m}In), technetium (^{99}Tc,^{99m}Tc), thallium (^{201}Ti), gallium (^{68}Ga, ^{67}Ga), palladium (^{103}Pd), molybdenum (^{99}Mo), xenon (^{133}Xe), fluorine (^{18}F), ^{153}Sm, ^{177}Lu, ^{159}Gd, ^{149}Pm, ^{140}La, ^{175}Yb, ^{166}Ho, ^{90}Y, ^{47}Sc, ^{186}Re, ^{188}Re, ^{142}Pr, ^{105}Rh, and ^{97}Ru.

[00104] In other embodiments, the antibody of the invention can be conjugated to a therapeutic agent. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis- dichlorodiamine platinum (II) (DDP)

cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), anti-mitotic agents (e.g., vincristine and vinblastine), and radionuclides. Examples of radionuclides useful as therapeutic agents include, but are not limited to, ^{131}I, ^{177}Lu, ^{90}Y, and ^{186}Re.

[00105] Techniques for conjugating such therapeutic moieties to antibodies are well-known, see, e.g., Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy," in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.*, eds., Alan R. Liss, Inc. (1985), pp. 243-256; Hellstrom *et al.*, "Antibodies For Drug Delivery," in *Controlled Drug Delivery*, 2nd Ed., Robinson *et al.*, eds., Marcel Dekker, Inc. (1987), pp. 623-653; Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera *et al.*, eds. (1985), pp. 475-506; "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy," in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.*, eds., Academic Press (1985), pp. 303-316; and Thorpe *et al.*, "The Preparation And Cytotoxic Properties of Antibody-Toxin Conjugates," *Immunol. Rev. 62*:119-158 (1982).

[00106] The present invention encompasses antibodies of the invention recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a polypeptide of interest to generate fusion proteins. The fusion does not necessarily need to be direct, but can occur through linker sequences. The antibodies of the present invention can be fused to either the N- or C-terminus of the heterologous protein (e.g., immunoglobulin Fc polypeptide or human serum albumin polypeptide). For example, antibodies can be fused to albumin, such as recombinant human serum albumin (see, e.g., U.S. Patent No. 5,876,969, EP 0 413 622, and U.S. Patent No. 5,766,883)), resulting in a chimeric polypeptide. In other embodiments, antibodies can be fused to the mature form of human serum albumin (i.e., amino acids 1–585 of human serum albumin as shown in

Figures 1 and 2 of EP Patent 0 322 094). In other embodiments, antibodies can be fused with polypeptide fragments comprising, or alternatively consisting of, amino acid residues 1-z of human serum albumin, where z is an integer from 369 to 419, as described in U.S. Patent No. 5,766,883. Antibodies fused or conjugated to polypeptides or other molecules of interest can also be used in *in vitro* immunoassays and purification methods using methods known in the art. See e.g., Harbor *et al., supra,* and PCT publication WO 93/21232; EP 439,095; Naramura *et al., Immunol. Lett. 39*:91-99 (1994); U.S. Patent No. 5,474,981; Gillies *et al., PNAS 89*:1428-1432 (1992); and Fell *et al., J. Immunol. 146*:2446-2452(1991).

[00107] The antibodies can be fused to marker sequences, such as a peptide to facilitate purification. In some embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al., Cell 37*:767 (1984)), and the "flag" tag (Stratagene, CA).

[00108] While the antibody conjugates of the invention can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as being limited to classical chemical therapeutic agents. For example, the drug moiety can be a protein or polypeptide possessing a desired biological activity. Such proteins can include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF-alpha, TNF-beta, AIM I (*see*, WO 97/33899), AIM II (*see*, WO 97/34911), Fas Ligand (Takahashi *et al., Int. Immunol. 6*:1567-1574 (1994)), VEGI (*see*, WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological response modifiers such as,

for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

[00109] Antibodies of the invention can also be attached to solid supports, which are useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride and polypropylene.

[00110] Alternatively, the antibody of the invention can be conjugated to a second antibody to form an antibody heteroconjugate as described, e.g., in U.S. Patent No. 4,676,980.

Assays For Antibody Binding

[00111] The antibodies of the invention can be assayed for immunospecific binding by any suitable method known in the art. The immunoassays that can be used include but are not limited to competitive and non-competitive assay systems using techniques such as BIAcore analysis, FACS (Fluorescence activated cell sorter) analysis, immunofluorescence, immunocytochemistry, Western blots, radio-immunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see e.g., Ausubel *et al*, eds, *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York (1994)). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

[00112] Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X- 100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease

inhibitors (e.g., EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (e.g., 1-4 hours) at 4°C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4°C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, e.g., Western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (e.g., pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols, see e.g., Ausubel *et al.*, eds, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York (1994), at 10.16.1.

[00113] Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8%-20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), incubating the membrane with primary antibody (the antibody of interest) diluted in incubating buffer, washing the membrane in washing buffer, incubating the membrane with a secondary antibody (which recognizes the primary antibody, e.g., an anti-human antibody) conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e.g., ^{32}P or ^{125}I) diluted in incubating buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding Western blot protocols, see e.g., Ausubel *et al.*, eds, *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York (1994) at 10.8.1.

[00114] ELISAs comprise preparing antigen, coating the well of a 96-well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound can be added to the well. Further, instead of coating the well with the antigen, the antibody can be coated to the well. In this case, a second antibody conjugated to a detectable compound can be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, e.g., Ausubel *et al.*, eds, *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York (1994) at 11.2.1.

[00115] The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., ^{3}H or ^{125}I), or fragment or variant thereof, with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for hTF and the binding off-rates can be determined from the data by Scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the hTF is incubated with the antibody of interest conjugated to a labeled compound (e.g., compound labeled with ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody. This kind of competitive assay between two antibodies can also be used to determine if two antibodies bind the same or different epitopes.

Blood Coagulation

[00116] Blood coagulation is a complex process involving three interacting components: blood vessels, blood coagulation factors, and blood platelets. Blood coagulation factors are proteins or glycoproteins present in the blood as inactive precursors. When bleeding occurs, the coagulation cascade is initiated and the inactive coagulation factors are converted to active proteases or enzymes.

[00117] Coagulation factors are activated in sequence in the coagulation cascade, with the aid of cofactors (such as calcium, TF, and phospholipids), resulting in the eventual formation of a fibrin clot. Fibrin is a sticky, thread-like protein that is insoluble in blood and provides the foundation for platelet adhesion and blood coagulation.

[00118] If bleeding results from an injury outside of the vasculature (such as an abrasion or cut of the skin), the extrinsic pathway is initiated. If injury occurs within the blood vessel itself, the intrinsic pathway is activated. Many bleeding episodes activate both pathways.

[00119] The extrinsic coagulation pathway is triggered on the extravascular cell surface when TF is exposed to blood following some physical injury. TF is a protein that can bind to both activated and inactivated forms of factor VII. In the extrinsic pathway, a small amount of circulating activated factor VII (factor VIIa) complexes with TF following its release. This TF/factor VIIa complex initiates coagulation by converting factors IX and X to active forms.

[00120] This reaction is amplified by a feedback mechanism in which factors VIIa, IXa and Xa activate additional factor VII bound to TF. Factor Xa, in complex with a cofactor, factor Va, and phospholipids, continues in the cascade activating prothrombin (also known as factor II) to thrombin (also known as factor IIa). Another feedback mechanism involving thrombin works to activate factors V, VIII and XI. Factor VIIIa complexes with factor IXa on platelet surfaces to activate factor X, resulting in more local thrombin generation. Thrombin is responsible for the eventual generation of fibrin.

[00121] In the intrinsic pathway, circulating activated factor XII, in complex with high molecular weight kininogen and prekallikrein, comes into contact with the exposed subendothelial membrane to initiate coagulation and activate factor XI. Factor XIa complexes with calcium to activate factor IX. Factor IXa, in conjunction with factor VIIIa, calcium and phospholipids, results in the activation of factor X to factor Xa and subsequent thrombin generation. After activation of factor X, the extrinsic and intrinsic pathways merge.

[00122] The final step of clot formation is the conversion of plasma soluble fibrinogen to insoluble fibrin as a result of the cleavage of peptide bonds. Cleavage occurs as the result of the proteolytic enzyme thrombin, which is produced from prothrombin. Conversion of prothrombin to thrombin requires a number of proteins called clotting factors, in addition to calcium. The fibrin clot is a crosslinked matrix, which entraps the formed elements of the blood thereby sealing off the site of bleeding. Formed elements consist of platelets, white blood cells, and red blood cells.

[00123] TF is a cell-anchored component that, together with factor VIIa, initiates blood coagulation *in vivo*. TF is a transmembrane glycoprotein with a 219 residue extracellular region, a 23 residue transmembrane region and a 21 residue cytoplasmic region. The extracellular region of TF has two fibronectin III-like domains and a distribution of disulfide bridges characteristic of class-II cytokine and interferon receptors. The cytoplasmic region of TF is short but contains at least one serine residue that can be phosphorylated. TF is also known as thromboplastin, factor III, and CD142.

[00124] TF forms a tight complex ($K_d \sim$ pmol) with its native ligand, i.e., factor VIIa. In the complex, VIIa wraps around TF (Banner, D.W., *et al., Nature 380*:41-46 (1996)) and forms an extensive region of contact with the TF surface. TF binds and allosterically activates factor VIIa (fVIIa) and the complex TF/fVIIa is responsible for thrombin generation via activation of factors IX and X and is the major initiator of blood clotting under physiological conditions. Antibodies that bind to the TF-VIIa interaction site can inhibit TF-VIIa interaction, thus inhibiting or blocking blood coagulation.

The antibodies of the present invention bind to TF, e.g., hTF, but do not inhibit TF mediated blood coagulation compared to a normal plasma control.

[00125] As used herein, the term "normal plasma control" means plasma pooled from normal human donors, such as that offered by George King Bio-Medical, Inc., Kansas (POOLED NORMAL PLASMA).

[00126] In some embodiments, the effect an antibody of the invention has on TF mediated blood coagulation can be determined using a blood clotting assay. For example, blood clotting assays known in the art, such as those described in, e.g., Morrissey, J.H., *et al.*, *Thrombosis Research 52*:247-261 (1988), and Fang, C.H., *et al.*, *Thrombosis and Haemostasis 76*:361-368 (1996), can be used to determine the effect of an anti-TF antibody on blood coagulation. Other blood clotting assays include, but are not limited to, one-stage prothrombin time assay (Miale J.B., *Laboratory Medicine, Hematology*, CN Mosbey Co., St. Louis (1977), and two-stage clotting assay (Bach *et al.*, *Biochemistry* 15:4007-20 (1986)) can also be used.

[00127] An antibody of the invention "does not inhibit TF mediated blood coagulation compared to a normal plasma control" where, in an hTF coagulation assay conducted as described in the Example section below, the clotting time of a blood sample treated with the antibody is about 150% or less, about 140% or less, about 130% or less, about 120% or less, about 110% or less, or about 100% or less of the clotting time of a normal plasma control.

Fc-Mediated Mechanisms

[00128] In some embodiments, the antibodies of the invention which are capable of binding to hTF without inhibiting TF mediated blood coagulation compared to a normal plasma control, can initiate one or more Fc-mediated mechanisms.

[00129] When antibodies are exposed to proteolytic enzymes such as papain or pepsin, several major fragments are produced. The fragments that retain antigen-binding ability consist of the two "arms" of the antibody's Y configuration and are termed F(ab) (fragment-antigen binding) or $F(ab')_2$

which represent two Fab arms linked by disulfide bonds. The other major fragment produced constitutes the single "tail" or central axis of the Y and is termed Fc (fragment-crystalline) for its propensity to crystallize from solution. The Fc fragment of IgG, IgA, IgM, or IgD consists of dimers of the two carboxyl-terminal domains of each antibody (i.e., CH2 and CH3 in IgG, IgA and IgD, and CH3 and CH4 in IgM). The IgE Fc fragment, by contrast, consists of a dimer of its three-carboxyl-terminal heavy chain domains (C2, C3 and C4).

[00130] The Fc fragment contains the antibody's biologically "active site," which enables the antibody to "communicate" with other immune system molecules or cells and thereby activate and regulate immune system defensive functions or host-mediated mechanisms. Such communication occurs when active sites within the antibody Fc region binds to molecules termed Fc receptors. Fc receptors are molecules that bind with high affinity and specificity to active sites within immunoglobulin Fc regions. Fc receptors can exist as integral membrane proteins within a cell's outer plasma membrane or can exist as free, "soluble" molecules that freely circulate in blood plasma or other body fluids.

[00131] For each of the five antibody classes, there are several types of Fc receptors that specifically bind to the Fc region of that class and perform distinct functions. Thus, IgE Fc receptors bind with high affinity to only IgE Fc regions or to isolated IgE Fc fragments. It is known that different types of class-specific Fc receptors exist, which recognize and bind to different locations within the Fc region. For example, certain IgG Fc receptors bind exclusively to the second constant domain of IgG (CH2), while Fc receptors mediating other immune functions bind exclusively to IgG's third constant domain (CH3). Other IgG Fc receptors bind to active sites located in both CH2 and CH3 domains and are unable to bind to a single, isolated domain.

[00132] Many of the functions of antibodies are mediated through their interaction with Fc receptors. These receptors are found on a variety of cells including macrophages, other leukocytes, platelets and placental trophoblasts.

[00133] After antibodies bind to antigens or are otherwise caused to aggregate, active sites within the Fc region are able to bind to and activate Fc receptors, providing a critical link between antibodies and the rest of the immune system. Fc binding to Fc receptors can thus be characterized as the "final common pathway" by which antibody functions are mediated. If an antigen-bound antibody does not bind to an Fc receptor, the antibody is unable to activate the other portions of the immune system and is therefore rendered functionally inactive.

[00134] The Fc region of the immunoglobulin binds to the Fc receptor and the complex can trigger a variety of responses depending on cell type. In the case of macrophages, the response can include phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC). Once activated by the binding of antibody Fc region active sites, Fc receptors mediate a variety of important immune killing and regulatory functions. For example, certain IgG Fc receptors mediate direct killing of cells to which the antibody has bound via its Fab arms (antibody-dependent cell-mediated cytotoxicity (ADCC)). Other IgG Fc receptors, when occupied by IgG, stimulate certain white blood cells to engulf and destroy bacteria, viruses, cancer cells or other entities by a process known as phagocytosis. Fc receptors on certain types of white blood cells known as B lymphocytes regulate their growth and development into antibody-secreting plasma cells.

[00135] Depending upon the particular type of Fc receptor to which an Fc portion of an antibody or active peptide fragment thereof binds, the peptide can either initiate or inhibit immune functions. Initiation can occur if the Fc receptor is of the type that becomes activated by the act of binding to an Fc region or, alternatively, if an Fc active site peptide stimulates the receptor. The type of initiation produced can include, but is not limited to, functions directly or indirectly mediated by antibody Fc region-Fc receptor binding.

[00136] The ability to initiate immune system functions, including those listed above, is known to be therapeutically useful in treating diseases such as infectious diseases caused by bacteria, viruses or fungi, conditions in which the immune system is deficient due either to congenital or acquired conditions,

cancer and many other afflictions of human beings or animals. Such immunostimulation is also useful to boost the body's protective cellular and antibody response to certain injected or orally administered substances administered as vaccines. This list is not intended to be all-inclusive, but merely provides representative examples of diseases or conditions in which immune stimulation has a recognized therapeutic usefulness.

[00137] As used herein, the term "Fc-mediated mechanism" refers to the initiation of an immune response to foreign antigens, mediated through Fc receptor activation. Fc-mediated mechanisms include, but are not limited to, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

[00138] In some embodiments, where the antibody of the invention can initiate Fc-mediated mechanisms, that mechanism is antibody-dependent cell-mediated cytotoxicity (ADCC). In yet other embodiments, the antibody of the invention can initiate complement-dependent cytotoxicity (CDC).

[00139] Antibody-dependent cell-mediated cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC) is a process by which natural killer cells, T lymphocytes, monocytes/macrophages and polymorphonuclear neutrophils (effector cells) are triggered to destroy foreign or infectious cells. IgG antibodies must first bind to antigens on the target cell, which sensitizes the cell for recognition by cells that mediate ADCC. Upon encounter with an IgG-sensitized target, IgG Fc receptors on cells that mediate ADCC bind to exposed Fc regions on the surface of the target cell. Such Fc receptor binding activates cells that mediate ADCC to directly lyse the target cell, causing its death. ADCC includes, but is not limited to, stimulation of phagocytosis by certain classes of white blood cells (polymorphonuclear neutrophils, monocytes and macrophages); macrophage activation; natural killer (NK) cell activity; growth and development of B and T lymphocytes and secretion by lymphocytes of lymphokines (molecules with killing or immunoregulatory activities).

[00140] Complement-dependent cytotoxicity (CDC) (or complement-mediated cytotoxicity, or complement-mediated cell lysis) is another process by which

foreign or infectious agents can be destroyed. An antibody interaction with a foreign antigen, forming an antibody-antigen complex, can result in a conformational change in the Fc region of the antibody. This conformational change may activate complement factor C1, thereby initiating a complement activation cascade involving complement initiation factors C1, C2, C3, and C4. The complement activation cascade terminates in the sequential interaction of C5, C6, C7, C8 and C9 forming the membrane-attack complex (MAC). MAC mediates cell lysis by disrupting the phospholipid membrane of a cell to form large pores in the cell membrane. See, e.g., Reff, M.E. *et al.* *Blood 83*:435-445 (1994). In this way, the MAC complex is capable of stimulating cell death of a foreign or infectious agent containing an antigen recognized by an antibody of the invention. In addition, C3 and C4 can act as peptide mediators of inflammation, a process that results in localized vasodilation and migration of neutrophils, macrophages and other phagocytic cells. These phagocytic cells can bear Fc receptors, thereby increasing localized antibody-dependent cellular cytotoxicity.

[00141] In some embodiments, the antibody of the invention contains moderate to high Fc-mediated activity, including, but not limited to, moderate to high ADCC and/or moderate to high CDC activity. An antibody of the invention has "moderate to high" ADCC activity if at an antibody concentration of 10 μg/ml and effector cell to target cell ratio of 30, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% of target cells are lysed. An antibody of the invention has "moderate to high" CDC activity if at an antibody concentration of 10 μg/ml and in the presence of undiluted human serum or rabbit serum, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% of target cells are lysed.

[00142] Any of the known assays in the art can be used to monitor the Fc-mediated mechanisms of the antibodies of the invention. The ability of the antibodies of the present invention to initiate one or more Fc-mediated mechanisms can be monitored *in vitro* or *in vivo*. For example, CDC activity

and ADCC activity of the antibodies can be measured by the methods of Ohta *et al., Cancer Immunol. Immunother. 36*:260 (1993). Other assays include but are not limited to a ^{51}Cr release assay of antibody-dependent cell-mediated cytotoxicity, and complement-mediate lysis can also be used. See *Current Protocols in Immunology*, Coligan, A.M. *et al.* (Eds.), Wiley & Sons, Inc. (1991), e.g., Unit 7.27; Wang, B. *et al., Proc. Natl. Acad. Sci. USA 96*:1627-1632 (1999); Manches, O. *et al., Blood 101*:949-954 (2003).

[00143] Furthermore, Fc-mediated host responses can be monitored *in vitro* by conventional immunoassays, where the anti-tumor activity of the response can be determined by CDC and/or ADCC assays. The assay methodologies are well known, and are described in Handbook of Experimental Immunology, Vol. 2, Blackwell Scientific Publications, Oxford (1986). In addition, CDC activity and ADCC activity of humanized chimeric antibody to a cultured cancer cell line can be measured in accordance with the procedures disclosed in Menekigaku Jikken Nyumon, (Manual of Immunological Experiments) Matsuhashi *et al.*, Gakkai Shuppan Center, Japan, 1981).

[00144] Fc-mediated mechanisms can be monitored *in vivo* by the development of delayed-type hypersensitivity reactions, or other *in vivo* or *in vitro* means known to those skilled in the art, including but not limited to the skin test reaction protocol, lymphocyte stimulation assays, measuring the toxicity of a subject's lymphocytes to tumor cells by using a standard radioactive release assay, by a limiting dilution assay, or by measuring plasma levels of IL-2 using standard ELISA assays.

Therapeutic Uses

[00145] The present invention is also directed to a method of treating cancer in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of the antibody of the invention. In some embodiments, this antibody-based therapy involves administering antibodies of the invention to an animal, more particularly a mammal, and more particularly a human patient, for treating cancer.

[00146] A "therapeutically effective amount" is an amount of a compound that, when administered to a subject or patient for treating a condition, disorder or disease, is sufficient to elicit a cellular response that is clinically significant, without excessive levels of side effects. See, "Formulations and Therapeutic Administration" section, infra, for further details.

[00147] "Subject" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, and companion animals such as a household pet and other domesticated animal such as, but not limited to, cattle, sheep, ferrets, swine, horses, poultry, rabbits, goats, dogs, cats and the like. In some embodiments, companion animals are dogs and cats. In other embodiments, the subject is human.

[00148] "Patient" refers to a subject, e.g., a human, in need of treatment of a condition, disorder or disease, e.g., cancer.

[00149] The terms "treat" and "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent, inhibit, or slow down (lessen) an undesired physiological condition, disorder or disease or obtain beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms; diminishment of extent of condition, disorder or disease; stabilized (i.e., not worsening) state of condition, disorder or disease; delay in onset, or slowing, of condition, disorder or disease progression; amelioration of the condition, disorder or disease state, remission (whether partial or total); or enhancement or improvement of condition, disorder or disease. Treatment also includes, but is not limited to, eliciting a cellular response that is clinically significant, without excessive levels of side effects. Treatment also includes, but is not limited to, prolonging survival as compared to expected survival if not receiving treatment.

[00150] Therapeutic compounds of the invention include, but are not limited to, antibodies of the invention and nucleic acids encoding antibodies of the invention. The antibodies of the invention can be used to treat disorders or conditions associated with cancer including, but not limited to, any one or more of the diseases, disorders, or conditions described herein. Antibodies of

the invention can be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

[00151] The terms "tumor" and "cancer" are used interchangeably, and, along with their grammatical variants, refer to tumors of any cell type, including carcinomas, sarcomas, lymphomas and leukemias of any human and non-human animal species including swine, cats, dogs and higher primates. The methods and compositions of the present invention are suitable for the treatment of solid tumors, which can be characterized by extensive vasculature (microvascularized tumors), including carcinomas, sarcomas and lymphomas of various cell types. Solid tumors targeted by the treatment of the present invention include, but are not limited to: cancers of head and neck, including squamous cell and epidermoid carcinomas; adenocarcinomas, including prostatic, scirrhous, and mammary adenocarcinomas; lymphosarcoma; fibrosarcoma; leiomyosarcoma; chondroma; cancer of the prostate, lung, breast, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, or thyroid; primary tumors and metastases; melanomas; glioblastoma; Kaposi's sarcoma; non-small cell lung cancer; advanced malignancies; and blood born tumors such as, e.g., leukemias.

[00152] Malignant and metastatic conditions that can be treated with the antibodies of the invention include, but are not limited to, malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman *et al.*, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia (1985)). Thus, antibodies of the invention can be useful in treating other diseases, disorders, and/or conditions, besides cancers, which involve angiogenesis. These diseases, disorders, and/or conditions include, but are not limited to: benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; artheroscleric plaques; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, uvietis and Pterygia (abnormal blood vessel growth) of the eye; rheumatoid arthritis;

psoriasis; delayed wound healing; endometriosis; vasculogenesis; granulations; hypertrophic scars (keloids); nonunion fractures; scleroderma; trachoma; vascular adhesions; myocardial angiogenesis; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; fibromuscular dysplasia; wound granulation; Crohn's disease; and atherosclerosis.

[00153] Treatment of metastases can be shown by the ability of the antibody of the invention to prevent tumor metastases in an animal model. For example, the spontaneous metastases model and the pulmonary metastases tumor model are metastases models known in the art. In the spontaneous metastases tumor model, an animal is subcutaneously injected with tumor cells which form a primary tumor mass. Subsequently, some of the cells of the tumor spontaneously migrate to other parts of the animal, including the lung. See Zisman, A. *et al.*, *Cancer Research 63:*4952-59 (2003); Lev, D.C. *et al., Clin. Exp. Metas. 20:*515-23 (2003). In the pulmonary metastases tumor model, a suspension of tumor cells is injected into the tail vein a mouse and the formation of metastases in the lungs of the recipient animal is evaluated. See Tian F. *et al, Cancer Research 63:*8284-92 (2003); Ogawa, K. *et al., Int. J. Cancer 91:*797-802 (2001). In these models, an antibody that is effective in the treatment of metastases will, upon its administration to the recipient animal, either prevent metastases from occurring or reduce the number of metastases that form as compared to the number of metastases formed in a recipient animal given a negative control.

[00154] The antibody of the invention can be used to treat and/or diagnose hyperproliferative diseases, disorders, and/or conditions, including neoplasms. The antibody can inhibit proliferation of the disorder through direct or indirect interactions. For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, differentiating, or mobilizing T-cells, hyperproliferative diseases, disorders, and/or conditions can be treated, and/or diagnosed. This

immune response can be increased by either enhancing an existing immune response, or by initiating a new immune response.

[00155] Examples of hyperproliferative diseases, disorders, and/or conditions that can be treated, and/or diagnosed by the antibodies of the invention, include, but are not limited to, neoplasms located in the colon, lung, abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic, and urogenital systems.

[00156] Similarly, other hyperproliferative diseases, disorders, and/or conditions can be treated and/or diagnosed by antibodies of the invention. Examples of such hyperproliferative diseases, disorders, and/or conditions include, but are not limited to, hypergammaglobulinemia, lymphoproliferative diseases, disorders, and/or conditions, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenstron's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

[00157] The present invention provides for treatment of diseases, disorders, and/or conditions associated with neovascularization by administration of the antibodies of the invention. The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate. Rastinejad *et al., Cell 56*:345-355 (1989). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases. A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye diseases, disorders and/or conditions, and psoriasis. See, e.g., reviews by

Moses *et al., Biotech. 9*:630-634 (1991); Folkman *et al., N. Engl. J. Med., 333*:1757-1763 (1995); Auerbach *et al., J. Microvasc. Res. 29*:401-411 (1985); Folkman, Advances in Cancer Research, eds. Klein and Weinhouse, Academic Press, New York (1985), pp. 175-203; Patz, *Am. J. Opthalmol. 94*:715-743 (1982); and Folkman *et al., Science 221*:719-725 (1983). In a number of pathological conditions, the process of angiogenesis contributes to the disease state. For example, significant data has been accumulated suggesting that the growth of solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, *Science 235*:442-447 (1987).

[00158] Additional ways in which the antibodies of the present invention can be used therapeutically include, but are not limited to, directed cytotoxicity of the antibody, e.g., as mediated by complement (CDC) or by effector cells (ADCC), or indirect cytotoxicity of the antibody, e.g., as immunoconjugates.

[00159] The antibodies of this invention can be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic growth factors (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies, or as conjugated to a cytotoxic agent such as a radioisotope or other cytotoxic agent as described above.

[00160] The antibodies of the invention can be administered alone or in combination with other types of treatments (e.g., radiation therapy, chemotherapy, hormonal therapy, immunotherapy, anti-tumor agents, and anti-retroviral agents). In some embodiments, antibodies of the invention can be administered alone or in combination with anti-retroviral agents.

Formulations, Therapeutic Administration, and Kits

[00161] The invention also provides methods of treatment by administration to a subject of an effective amount of a compound, e.g., an antibody of the invention, or a pharmaceutical composition of the invention. In some embodiments, the antibody is substantially purified (e.g., substantially free

from substances that limit its effect or produce undesired side-effects). The antibody can be conjugated to a cytotoxic agent.

[00162] Formulations and methods of administration that can be employed when the compound comprises an immunoglobulin are described herein; additional appropriate formulations and routes of administration can be selected from among those described herein below.

[00163] Various delivery systems are known and can be used to administer the compound or pharmaceutical composition of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see e.g., Wu and Wu, *J. Biol. Chem. 262*:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions can be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and can be administered together with other biologically active agents. Administration can be systemic or local. In addition, it can be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection can be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

[00164] In some embodiments, it can be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this can be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes,

such as sialastic membranes, or fibers. When administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not adsorb.

[00165] In other embodiments, the compound or composition can be delivered in a vesicle, in particular a liposome (see, Langer, *Science 249*:1527-1533 (1990); Treat *et al.*, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler, eds., Liss, New York (1989), pp. 353-365; Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

[00166] In yet other embodiments, the compound or composition can be delivered in a controlled release system. In some embodiments, a pump can be used (see Langer, supra; Sefton, *CRC Crit. Ref. Biomed. Eng. 14*:201 (1987); Buchwald *et al., Surgery 88*:507 (1980); Saudek *et al., N. Engl. J. Med. 321*:574 (1989)). In other embodiments, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise, eds., CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball, eds., Wiley, New York (1984); Ranger and Peppas, *J., Macromol. Sci. Rev. Macromol. Chem. 23*:61 (1983); see also Levy *et al., Science 228*:190 (1985); During *et al., Ann. Neurol. 25*:351 (1989); Howard *et al., J. Neurosurg. 71*:105 (1989)). In yet other embodiments, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled release systems are discussed in the review by Langer, *Science 249*:1527-1533 (1990).

[00167] The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound of the invention, and a pharmaceutically acceptable carrier. In some embodiments, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized international pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is

administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. In some embodiments, water can be used as a carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences* by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[00168] In other embodiments, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition can also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette

indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

[00169] The amount of the compound of the invention, which will be therapeutically effective in the treatment of the disease or disorder, e.g., cancer, can be determined by standard clinical techniques. In addition, *in vitro* assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses can be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[00170] For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. However, for radiolabeled antibodies, the dosage administered can be lower, e.g., 0.01 mg/kg to 1 mg/kg of the patient's body weight, and for toxin-immunoconjugates, the dosage administered can be even lower, e.g., 0.001 mg/kg of the patient's body weight. In some embodiments, the dosage administered to a patient is between 0.001 mg/kg and 100 mg/kg of the patient's body weight. In other embodiments, the dosage administered to a patient is between 0.01 mg/kg and 50 mg/kg of the patient's body weight. In other embodiments, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight. In yet other embodiments, the dosage administered to a patient is between 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention can be reduced by enhancing

uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

[00171] The invention also provides a kit comprising the pharmaceutical composition of the present invention. The kit can include one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice or printed instructions.

[00172] For example, such printed instructions can be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of the manufacture, use or sale for human administration to treat a condition such as cancer. In some embodiments, the kit further comprises printed matter, which, e.g., provides information on the use of the pharmaceutical composition to treat cancer or a pre-recorded media device which, e.g., provides information on the use of the pharmaceutical composition to treat cancer, or a planner.

[00173] "Printed matter" can be, for example, one of a book, booklet, brochure or leaflet. The printed matter can describe the use of the pharmaceutical composition of the present invention for the treatment of cancer. Possible formats included, but are not limited to, a bullet point list, a list of frequently asked questions (FAQ) or a chart. Additionally, the information to be imparted can be illustrated in non-textual terms using pictures, graphics or other symbols.

[00174] "Pre-recorded media device" can be, for example, a visual media device, such as a videotape cassette, a DVD (digital video disk), filmstrip, 35 mm movie or any other visual media device. Alternately, pre-recorded media device can be an interactive software application, such as a CD-ROM (compact disk-read only memory) or floppy disk. Alternately, pre-recorded media device can be, for example, an audio media device, such as a record, audiocassette or audio compact disk. The information contained on the pre-recorded media device can describe the use of the pharmaceutical composition of the present invention for the treatment of cancer.

[00175] A "planner" can be, for example, a weekly, a monthly, a multi-monthly, a yearly, or a multi-yearly planner. The planner can be used as a diary to monitor dosage amounts, to keep track of dosages administered, or to prepare for future events wherein taking a regularly administered pharmaceutical composition of the present invention may be difficult. Alternately, the planner can be a calendar which will provide a means to monitor when a dosage has been taken and when it has not been taken. This type of planner will be particularly useful for patients having unusual schedules for administering medication to themselves. Additionally, the planner can be useful for the elderly, children, or other patient group who may administer medication to themselves and may become forgetful. One skilled in the art will appreciate the variety of planning tools that would be appropriate for use with the present invention.

[00176] The kit can also include a container for storing the other components of the kit. The container can be, for example, a bag, box, envelope or any other container that would be suitable for use in the present invention. Preferably, the container is large enough to accommodate each component and/or any administrative devices that may be necessary of the pharmaceutical composition of the present invention. However, in some cases, it may be desirable to have a smaller container which can be hidden in a patient's pocketbook, briefcase or pocket.

Methods of Delivering the Pharmaceutical Composition of the Present Invention to a Patient

[00177] The present invention is also directed to a method of delivering a pharmaceutical composition comprising a therapeutically effective amount of the antibody of the present invention to a patient in need thereof, the method comprising (a) registering in a computer readable medium the identity of a physician permitted to prescribe the pharmaceutical composition; (b) providing the patient with counseling information concerning the risks attendant to the pharmaceutical composition; (c) obtaining informed consent

from the patient to receive the pharmaceutical composition despite the attendant risks; (d) registering the patient in a computer readable medium after obtaining their informed consent; and (e) permitting the patient access to the pharmaceutical composition.

[00178] The drug delivery methods of the present invention involve, inter alia, registering in a computer readable storage medium physicians who are qualified to prescribe the pharmaceutical composition of the present invention. Once registered in the computer readable storage medium, the physician can be eligible to prescribe the pharmaceutical composition to a patient in need thereof. Generally speaking, in order to become registered in the computer readable storage medium, the physician may be required to comply with various aspects of, for example, providing patient education and counseling. The registration of the physician in the computer readable storage medium can be achieved by providing the physician, for example, by mail, facsimile transmission, or on-line transmission, with a registration card or form, preferably together with educational materials concerning the pharmaceutical composition of the present invention. The physician can complete the registration card or form by providing information requested therein, and the registration card or form can be returned to the manufacturer or distributor of the pharmaceutical composition of the present invention, or other authorized recipient of the registration materials, for example, by mail, facsimile transmission or on-line transmission. The physician's information in the registration card or form is then entered into the computer readable storage medium. Suitable computer readable storage media which can be employed for registration of the physicians (as well as patients, as discussed below) will be apparent to one of ordinary skill in the art, once in possession of the teaching of the present application.

[00179] In the course of examination of a patient, including a patient suffering from cancer, the physician may determine that the patient's condition can be improved by the administration of the pharmaceutical composition of the present invention. Prior to prescribing the pharmaceutical composition of the present invention, the physician can counsel the patient, for example, on the

various risks and benefits associated with the pharmaceutical composition. The patient can be provided full disclosure of all the known and suspected risks associated with the pharmaceutical composition. Such counseling can be provided verbally, as well as in written form. In some embodiments, the physician can provide the patient with literature materials on the pharmaceutical composition, such as product information, educational materials, and the like.

[00180] In addition to receiving counseling on the risks attendant to the pharmaceutical composition of the present invention, the methods of the invention further require the patient to fill out an informed consent form which is signed by the patient. Upon the completion of the informed consent form, the patient can be registered in a computer readable storage medium. The computer readable storage medium in which the patient is registered can be the same as, or different from, the computer readable storage medium in which the physician is registered.

[00181] The registration into one or more computer readable storage media of the physician and patient, according to the methods describe herein, provides a means to monitor and authorize access to the pharmaceutical composition of the present invention. Thus, the computer readable storage medium can serve to deny access to patients who fail to abide by the methods of the present invention. In some embodiments, access to the pharmaceutical composition of the invention is in the form of a prescription, wherein the prescribing physician is registered in a computer readable storage medium, has provided counseling to the patient concerning the attendant risks of the pharmaceutical composition, and has obtained informed consent from the patient, prior to prescribing the pharmaceutical composition to the patient in need thereof.

Methods of Educating a Consumer Regarding the Pharmaceutical Composition of the Present Invention

[00182] The present invention is also directed to methods of educating consumers about the use of a pharmaceutical composition of the invention, the

method comprising distributing the pharmaceutical composition with consumer information at a point of sale. In some embodiments, the distribution will occur at a point of sale having a pharmacist or healthcare provider.

[00183] As used herein, the term "consumer information" can include, but is not limited to, an English language text, non-English language text, visual image, chart, telephone recording, website, and access to a live costumer service representative. In some embodiments of the present invention, consumer information will provide directions for use of the pharmaceutical composition of the present invention, appropriate age use, indications, contraindications, or warnings. In some embodiments, the method further comprises providing professional information to relevant persons in a position to answer consumer questions regarding the pharmaceutical composition.

[00184] As used herein, the term "professional information" includes, but is not limited to, information concerning the pharmaceutical composition of the present invention designed to enable a healthcare professional to answer costumer questions regarding the pharmaceutical composition.

[00185] A "relevant person," as used herein, includes, for example, a physician, physician assistant, nurse practitioner, pharmacist and customer service representative.

[00186] The present invention is further directed to a method of identifying a pharmaceutical composition comprising a therapeutically effective amount of the antibody of the invention, the method comprising (a) isolating an antibody capable of binding to human tissue factor, wherein the antibody does not inhibit tissue factor mediated blood coagulation compared to normal plasma controls and can initiate an Fc-mediated mechanism; (b) repeating (a) to obtain a plurality of candidate antibodies that may prove therapeutically effective; (c) demonstrating that one such candidate antibody is non-toxic when administered to a non-human animal; (d) conducting a supervised clinical trial to demonstrate the non-toxic and effective character of one such candidate antibody; (e) securing approval of a regulatory agency to distribute

one such candidate antibody to treat cancer; and (f) making a pharmaceutical composition comprising the candidate antibody as the active agent.

[00187] The phrase "isolation of antibody," as used herein, includes the use of assays and protocols, as previously described herein, concerning the production and isolation of an antibody capable of binding to human TF (hTF), wherein the antibody does not inhibit TF mediated blood coagulation compared to a normal plasma control as determined by in vitro coagulation assays. Upon the isolation of a plurality of candidate antibodies, the method further comprises demonstrating for a candidate antibody its non-toxic nature when administered to a non-human animal.

[00188] Methods for demonstrating the non-toxic nature of a pharmaceutical are well known in the art and include, but are not limited to, administering to a non-human animal the pharmaceutical composition of the present invention and conducting standard medical tests to establish the non-toxic effects of the pharmaceutical composition on the non-human animal receiving the pharmaceutical composition. In some embodiments of the present invention, the methods further comprise in vivo experimentation on non-human animals establishing the ability of the candidate antibody to bind human tissue factor, wherein the candidate antibody does not inhibit tissue factor mediated blood coagulation compared to normal plasma controls and can initiate an Fc-mediated mechanism in the non-human animal model.

[00189] A "clinical trial," as used herein refers to testing of a candidate antibody to evaluate its safety, determine the proper dosage range, and identify potential side effects of its use in humans. Additionally, clinical trials include studies conducted to confirm the pharmaceutical composition's effectiveness in treating cancer, as well as to provide information used to optimize the safe administration of the pharmaceutical composition to a human. Upon the successful completion of the clinical trial, the method further comprises securing the approval of a regulatory agency, e.g., the Food & Drug Administration, to make and distribute the candidate antibody for the treatment of cancer.

[00190] All of the various embodiments or options described herein can be combined in any and all variations.

[00191] The following examples are further illustrative of the present invention, but are not to be construed to limit the scope of the present invention.

EXAMPLES

Materials

[00192] Cell culture reagents were purchased from Invitrogen Corp., CA. Titan one Tube RT-PCR system was from Roche (Basel, Switzerland, Cat. # 1 855 476). Ni-NTA agarose was obtained from Qiagen (CA, Cat. # 30210) and Bio-GelP60 was from Bio-Rad (CA, Cat. # 150-4161). HiTrap protein G HP columns were purchased from Amersham (Buckinghamshire, United Kingdom, Cat. # 17-0404-01). Mouse anti-human TF mAb was obtained from Calbiochem (CA, Cat. # 612161). Pooled normal human plasma was from George King Bio-Medical Inc. (KA, Cat. # 0010-1). Cell dissociation solution was from Sigma (MO, Cat. # C-5914).

Construction of hTF expression vectors

[00193] The human tissue factor (hTF) gene was cloned from the human breast cancer cell line SKBR3 by RT-PCR. Briefly, 1 μg of total RNA was isolated from SKBR3 cells using the Trizol reagent (Invitrogen Corp., CA, Cat. # 15596018) according to the manufacturer's instructions. The isolated RNA was reverse-transcribed and amplified with primer TF4 (5' UTR-ACGGAACCCGCTCGATCTCG (SEQ ID NO:13)) and TF5 (3' UTR-TGCAGTAGCTCCAACAGTGC (SEQ ID NO:14)) using the Titan one Tube RT-PCR system following the manufacturer's instructions. The first PCR product was further amplified using primers TF1 (5'-ATC TGC GGA TCC ACC ATG GAG ACC CCT GCC TGG CC-3' (SEQ ID NO:15)) and TF3 (5'-ATC TGC CTC GAG TTA ATG GTG ATG GTG ATG GTG GGA TCC

TCT TTC TCT GAA TTC CCC TTT CTC CTG-3' (SEQ ID NO:16)) to generate a hTF DNA fragment encoding the extracellular domain of hTF with a 32 amino acid N-terminal leader sequence and a 9 amino acid C-terminus RGS-His_6 tag sequence (soluble hTF). The soluble hTF contained 5' BamHI and 3' XhoI sites for insertion into expression vectors including a His- tag at the 3' end for protein purification. The first PCR product was also used to generate a full-length hTF gene with primers TF1 and TF2 (5'-ATC TGC CTC GAG TTA ATG GTG ATG GTG ATG GTG GGA TCC TCT TGA AAC ATT CAG TGG GGA GTT CTC-3' (SEQ ID NO:17)). The amplified soluble hTF and full-length hTF were cloned into the pCR4-TOPO vector (Invitrogen Corp., CA) for sequence analysis. The soluble hTF (SEQ ID NO:3) and full-length hTF (SEQ ID NO:1) encoding DNA fragments were also cloned into pCEP4 and pcDNA3.1 expression vectors (Invitrogen Corp., CA).

Expression and purification of soluble hTF

[00194] $3x10^5$ cells/well of HEK293 cells were plated in a 6-well plate one day before transfection. Cells were transfected with 1 μg of soluble hTF/pCEP4 plasmid DNA using Lipofectamine plus reagent (Invitrogen Corp., CA) for 3 hours at 37°C according to the manufacturer's instructions. Stably transfected cells were selected by culturing cells in DMEM medium containing G418 (750 μg/ml). Soluble hTF protein was then purified from 300 ml of culture medium using a 1 ml size Ni-agarose column and eluted with linear imidazole buffer gradient (between 5 mM and 100 mM imidazole in PBS buffer). The soluble hTF containing fractions were identified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The soluble hTF was further purified by gel filtration chromatography to remove contaminants. Briefly, samples were concentrated to about 100 μl with a Centrifugal Filter (Millipore, MA, Cat. # UFV4BGC25) and loaded onto a 0.7 x 50 cm Bio-Gel P60 column in PBS. The protein was then eluted in PBS and 0.5 ml fractions were collected and analyzed by SDS-PAGE. The soluble hTF band was verified by standard

Western blotting using a mouse anti-hTF mAb (Calbiochem, CA, Cat. # 612161). The fractions containing soluble hTF were combined.

Preparation of stable full-length hTF expression cell line

[00195] CHO-K1 cells were transfected with the full-length hTF/pcDNA3.1 plasmid (pTF103) using Lipofectamine plus reagent following the manufacturer's instructions (Invitrogen Corp., CA). Clones stably expressing full-length hTF were selected in the presence of 750 μg/ml of G418 containing DMEM medium. After one week of selection, resistant cells were removed from plates with Trypsin-EDTA solution and diluted with DMEM/G418 medium to a concentration of 3 cells/ml. 100 μl aliquots of the dilution were added into each well of one 96-well plate. Single cell clones were expanded and screened by FACS using a commercial anti-TF antibody (Calbiochem, CA, Cat. # 612161).

Immunization and measurement of polyclonal, anti-hTF IgG response in mice

[00196] A protocol for the rapid immunization of mice at multiple sites (RIMMS) has been previously described by Kilpatrick, K.E., *et al.*, *Hybridoma* 16:381-389 (1997), and was used to generate antibodies against hTF in mice. Briefly, each of three 8-week-old, female Balb/c mice received 4 rounds of subcutaneous injections of the purified soluble hTF (10 μg/ml) over a course of 11 days at intervals of 3-5 days. For each round of immunization, the mice were anesthetized and then injected subcutaneously with the immunogen in complete Freund's adjuvant (CFA) at 2 sites in the nape of the neck and bilaterally at the calf and groin injecting 40-50 μl per site and in RIBI's adjuvant (Sigma, St. Louis, MO) at juxtaposed sites (lower and mid calf, thigh, and axilla), at a dose of 40-50 μl per site. Blood samples were taken prior to the priming injections and two days after the last boost and assayed in an ELISA for an antibody response to the immunogen. In the ELISA, a 96-well ELISA plate was coated with the immunogen (2 μg/ml) at 100 μl/well in PBS, pH 7.4 at 37°C for 2 hrs. The plate was washed once with

PBS containing 0.05% Tween-20 (PBS-T) and blocked with 1% BSA in PBS at 150 μl/well at 37°C for 30 min. Following one wash with PBS-T, preimmune and immune sera diluted in PBS-T were added to the plate at 100 μl/well. The plate was incubated at 37°C for 45 min and washed three times with PBS-T. Then, 100 μl of a 1:5000 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotech, Birmingham, Alabama, Cat. # 1031-05) was added to each well. Following incubation at 37°C for 30 min., the plate was washed with PBS-T three times. The antibody binding was visualized by adding 100 μl/well of TMB-H_2O_2 substrate buffer (Pierce, Rockford, IL). The reaction proceeded at room temperature for 10 min and was read using an ELISA plate reader at a wavelength of 650 nm. An antibody titer was defined as the reciprocal of serum dilution at which the O.D. reading was 2-fold higher than that of an internal negative control (wells with the secondary antibody only).

[00197] Antibodies TF278, TF277, TF392, and TF9 were generated using the same methods as described *supra*, except that each of the three Balb/c mice received 5 rounds of subcutaneous injections of the purified soluble hTF (10 μg/ml) over a course of 11 days at intervals of 2-4 days.

Generation of hybridomas

[00198] Two days after the final boost, the immunized mice whose sera had ELISA titers greater than 1:10,000 were euthanized by asphyxiation with carbon dioxide. Bilateral popliteal, superficial inguinal, axillary and branchial lymph nodes were isolated and washed with fresh medium containing penicillin and streptomycin. Then, a single cell suspension was prepared from the lymph nodes in serum-free medium composed of 50% Excell-610 and 50% RPMI-1640 media. The lymph node cell suspension was washed twice with the aforementioned medium and collected by centrifugation at 400 x g for 10 minutes at room temperature. In a 50-ml conical polypropylene tube, the lymph node cells were then fused with mouse myeloma cells (P3X63/Ag8.653, ATCC, Manassas, VA) at a ratio of 2.5:1 by adding 1 ml of

50% polyethylene glycol 1500 (PEG, Roche Bioscience, Palo Alto, CA). The resulting PEG-cell preparation was washed once and then resuspended in hybridoma medium (HM) containing a mixture of 50:50 Excell-610 and RPMI-1640, 10% FBS, 10% Origen Cloning Factor (Igen, Rockville, MD), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin and 0.01 mM beta-mercaptoethanol, and distributed into flat-bottom, 96-well plates at 2 X 10^5 cells/100 μl/well. After incubating for 18 hours at 37°C with 7% CO_2, 100 μl of HM supplemented with 2X HAT (GIBCO-BRL, Grand Island, NY) was added to each well. Media were changed 96 hr later to HM supplemented with 100 μM hypoxanthine and 16 μM thymidine. After 7 to 10 days of HAT selection, the plates were examined microscopically for hybridoma growth. Hybridomas from single colonies were further expanded individually in 24-well plates and the culture supernatants were screened by ELISA for mouse IgG antibodies specific for hTF (see below).

Primary screening for anti-hTF mAbs by ELISA

[00199] Briefly, a 96-well ELISA plate was coated with 100 μl/well of 2 μg/ml soluble hTF in PBS, pH 7.4 at 37°C for 2 hrs. The plate was washed once with PBS-T and blocked with 150 μl/well of PBS containing 1% BSA at 37°C for 30 min. Following washing once with PBS-T, hybridoma supernatants were added to the plate at 100 μl/well. The plate was incubated at 37°C for 45 min and washed three times with PBS-T. Then, 100 μl of a 1:5000 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotechnology, Cat. # 1031-05) was added to each well. Following incubation at 37°C for 30 min., the plate was washed with PBS-T three times. Antibody binding was visualized by adding 100 μl of TMB-H_2O_2 substrate buffer to each well. The reaction proceeded at room temperature for 10 min and was read using an ELISA plate reader at a wavelength of 650 nm. A positive reaction was defined as an O.D. reading, which was 2-fold higher than that of an internal negative control (secondary Ab alone). All ELISA positive clones were further expanded in HM and cryopreserved.

Secondary screening for anti-hTF mAbs by flow cytometry

[00200] Cells (CHO-K1) expressing full-length hTF were dissociated with 0.25% trypsin-EDTA solution, washed twice in cold PBS containing 2% FBS and 0.05% NaN_3 (FACS buffer) at 400 x g for 10 min. and then distributed into U-bottom, 96-well microtiter plates at 0.5 x 10^6 cell/well. The cells were centrifuged at 200 x g at 4°C for 3 min. Following removal of supernatants by aspiration, the cells were resuspended in 70 µl of hybridoma supernatant. After incubation at 4°C for 45 min., the cells were washed twice with cold FACS buffer, 220 µl/well, by centrifugation at 200 x g for 3 min and resuspended in 50 µl of 1:25 FITC-labeled goat anti-mouse IgG (Southern Biotechnology). The cells were incubated at 4°C for 30 min and then washed three times with cold FACS buffer, 220 µl/well. Finally, cells were resuspended in 0.4 ml of FACS buffer, and their fluorescence intensities were measured on a flow cytometer (FACScan, Becton Dickinson) and analyzed using Cell Quest software (Becton Dickinson). A positive clone was identified as a clone where the percent positive cells in the FACS profile was at least 3-fold higher than the profile obtained when the cells were stained only with the FITC-labeled goat anti-mouse IgG.

BIAcore analysis of anti-hTF mAbs

[00201] Binding properties of anti-hTF mAbs were evaluated using BIAcore X. Briefly, a CM5 BIAcore biosensor chip was docked into the instrument and activated with 55 µl of 1:1 NHS/EDC at room temperature. The recombinant soluble hTF and BSA (10 µg/ml in 0.05 M acetate buffer, pH 4.5) were immobilized on the activated chips in flow cells 1 and 2, respectively. The immobilization was carried out at a flow rate of 5 µl/min until a resonance response of 1000 – 2000 RU was achieved. The chip was then blocked by injection of 55 µl of ethanolamine-HCl, pH 8.5 followed by 5 washes with 50 mM NaOH, 1 M NaCl. To measure the binding of anti-hTF mAbs to the soluble hTF immobilized to the chip, 30 µl of anti-hTF mAbs at varying

concentrations in BIAcore running buffer (HBS-EP, Biacore AB, Uppsala, Sweden, Cat. #1001-08) were injected over the sensor surface at a flow rate of 5 μl/min. Following completion of the injection phase, dissociation was monitored in BIAcore running buffer at the same flow rate for 360 seconds. The surface was regenerated between injections using 30 μl of 50 mM NaOH-1 M NaCl. Individual sensorgrams were analyzed using BIAsimulation software. Representative data are shown in Tables 3 and 4.

Preparation of TF membrane extract

[00202] CHO-K1 cells (5 x 10^7) expressing full-length hTF were harvested with cell dissociation solution and washed once with ice-cold 1X PBS. Cells were resuspended in 2 ml of membrane extraction buffer (10 mM Tris-HCl, pH=8.0, 1 mM MgC12, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin) and homogenized on ice with a Tissue Tearor tissue homogenizer (Biospec Products, Inc., Bartlesville, OK) three times for 30 seconds each. Cell debris was removed by centrifugation at 1500 x g for 5 min. Cell membranes were collected by centrifugation at 12,000 x g for 30 minutes at 4°C. The pellets were resuspended in 1x PBS, aliquoted and stored at –20°C.

Purification of hybridoma IgG

[00203] ELISA and FACS positive hybridoma clones were cultured in low IgG hybridoma medium (40% RPMI1640, 40% EX-Cell Hybridoma medium, 10% low IgG FBS, 10% ORIGEN cloning factor, 2 mM L-glutamine, 10mM HEPES, 1mM Sodium Pyruvate) at 37°C, in a humidified atmosphere with 7% CO_2. 40 ml of culture medium containing the secreted antibody were loaded onto a 1 ml HiTrap protein G HP column and then washed with 10 ml of PBS. The bound IgG was eluted from the column with 3 ml of 0.1 M glycine, pH 3.7 and neutralized with 1 M Tris-HCl, pH 9.0. The fractions containing IgG were pooled and dialyzed in PBS.

hTF coagulation assay

[00204] Anti-coagulation activity of the hTF antibodies was determined using an assay according to Morrissey, J.H., *et al.*, *Thrombosis Research* 52:247-261 (1988) (also known as a two stage prothrombin (2st-PT) assay), and Fang, C.H., *et al.*, *Thrombosis and Haemostasis* 76: 361-368 (1996). Different dilutions of the hTF membrane extract were adjusted with PBS to 100 μl and pre-warmed in a 37°C water bath for 30 minutes. 50 μl of human plasma and 50 μl of 50 mM $CaCl_2$ solution were then added to the mixture to initiate blood coagulation in a clear disposable plastic cuvette. Blood coagulation was monitored by measuring in 15 second intervals the absorbance at 405 nm (A_{405}). Blood coagulation was complete when the change in the A_{405} reading reached less than 0.01 in 15 seconds. The hTF membrane dilution that resulted in a blood coagulation time of 180 seconds was used to test the inhibitory effect of the hTF mAbs. To test the inhibitory effect of hTF mAb on blood coagulation, hTF membrane extract was incubated with each mAb (final concentrations of 10 μg/ml to 100 μg/ml) at 37°C for 30 min before the initiation of the blood coagulation reaction.

Table 1. Summary for Screening of Anti-hTF Hybridomas by ELISA and FACS

	Number of Clones	Percentage
# of clones screened	253	N/A
# of ELISA-positive clones	102	40.3
# of FACS-positive clones	31	12.3

Table 2. Summary for Individual FACS-positive Clones

Clone ID	% Positive
11	99.1
20	99.5
28	99.4
29	99.7

Clone ID	% Positive
41	99.5
54	98.0
75	99.3
84	99.4
85	97.3
92	73.3
120	36.5
128	99.8
129	98.1
144	99.3
169	97.9
170	99.5
172	99.5
196	99.8
199	99.8
236	99.2
248	67.0
260	99.6
109	32.6
130	28.1
112	26.6
50	25.5
68	16.1
12	13.7
251	13.3
93	11.9
190	11.6

Note: The results were obtained by FACS screening of culture supernatants of individual hybridomas grown in 24-well plates. Percent positive indicates the population of TF34 cells that stably expressed TF and stained positive.

Table 3. Summary of Selected Monoclonal Anti-TF Antibodies

Clone#	FACS	TF coagulation (s)	Ka (1/Ms)	Kd (1/s)	KD=kd/ka (M)
# 260	+	190 ± 9	9.91E+03	1.91E-04	1.93E-08
# 196	+	195 ± 0	4.21E+04	2.79E-03	6.63E-08
# 236	+	217 ± 11	2.62E+04	1.86E-02	7.10E-07
# 54	+	225 ± 0	1.11E+05	3.84E-02	3.46E-07
# 84	+	400 ± 28	8.18E+04	1.02E-03	1.25E-08
Normal Control		191 ± 16			
B-Fact (Borderline)		240			

Note: Normal plasma control and B-Fact control (pooled normal human plasma diluted to 30-50% of normal coagulation activity (George King Bio-medical, Inc., KS, Cat. #0040-0)) showed the blood coagulation time for normal human blood and borderline human blood samples in our assay format. All antibodies that had shorter coagulation times than the borderline control are listed. Only one representative inhibitory antibody (#84) with longer coagulation time is shown. Most of the antibodies inhibit coagulation.

Table 4. Summary of Selected Monoclonal Anti-TF Antibodies

Clones	**Isotype**	**Ab Con.**	**Coagulation**	**Kd (1/s)**	**KD (M)**	**FACS (H596)**	
		(ug/ml)	**(seconds)**			**Bmax**	EC50 (nM)
TF278	IgG1λ	10	190±17.3 (3)	1.38E-04	7.89E-10	16, 26	0.48, 1.14
TF9	IgG1k	10	195±15 (3)	3.64E-04	1.84E-09		
TF392	IgG1λ	10	210	1.30E-04	2.46E-09	58	6 .29
TF277	IgG1k	10	202.5±10.6 (2)	3.12E-03	7.14E-09	19, 29	0.48, 0.71

Note: In Table 4, the same normal plasma control and B-Fact control (pooled normal human plasma diluted to 30-50% of normal coagulation activity (George King Bio-medical, Inc., KS, Cat. #0040-0)) as displayed in Table 3

were used. All antibodies listed in Table 4 had a shorter coagulation time than the borderline control (B-Fact control).

ADCC Activity

[00205] The ADCC activity of anti-TF antibodies TF260, TF278, and TF392 was determined using the following ADCC assay. Human leukocytes were isolated from peripheral blood of normal donors using a Histopaque-1077 gradient centrifugation procedure (Sigma Co., St. Louis, MO). The isolated leukocytes were then used as effector cells. In U-bottom, 96-well plates, tumor cells (5×10^3 /well) were mixed with the Histopaque-purified human leukocytes at effector-to-target (E/T) ratios of 0:1 – 40:1 in the absence or presence of varying concentrations of anti-human TF mAbs or a control antibody in a total volume of 120 µl of RPMI 1640 supplemented with 10% FBS. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Target cells mixed with effector cells without the testing antibody were used as negative controls. Following a 16 – 18 hr. incubation, 50 µl aliquots of culture supernatant were collected and assayed for lactate dehydrogenase activity in flat-bottom, 96-well plates using the Cytotox 96 Non-radioactive Cytotoxicity Assay Kit (Promega Co., Madison, WI) according to the manufacturer's instruction. The percentage lysis of tumor cells was calculated as follows: % Cytotoxicity = experimental release – effector spontaneous release – target spontaneous release)/(target maximum release – target spontaneous release) x 100. The ADCC results for anti-TF antibodies TF260, TF278, and TF392 were expressed as mean percentage lysis ± S.D. of triplicate samples from 6 – 7 donors and can be found in FIGS. 4A-4C. For the ADCC assays, TF-positive SW900 and TF-negative A549 lung tumor cells were used as targets. An irrelevant human IgG1 was used as the negative antibody control. FIGS. 4A-4C demonstrate that anti-TF antibodies TF260, TF278, and TF392 cause an increase in % cytotoxicity when incubated with TF-positive cells as compared to the negative antibody control (hIgG).

[00206] All documents, e.g., scientific publications, patents and patent publications, recited herein are hereby incorporated by reference in their entirety to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference in its entirety. Where the document cited only provides the first page of the document, the entire document is intended, including the remaining pages of the document.